MPHIL

Development of Selective Inhibitors of Dihydrofolate Reductase (DHFR) of Mycobacterium Tuberculosis

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Development of selective inhibitors of dihydrofolate reductase (DHFR) of *Mycobacterium tuberculosis*

submitted by

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Abstract

Mycobacterium tuberculosis is the causative organism for one of the pandemic diseases in the world, tuberculosis (TB). The length of treatment often results in multi-drug resistance (MDR) and patient non-compliance. One of the most important enzymes as a drug target for tuberculosis is dihydrofolate reductase (DHFR), which plays an important role in the folate cycle and inhibition of the enzyme stops cell growth. DHFR inhibitors are usually 2,4-diaminopyrimidines, which have high binding affinity to the enzyme but have the potential to inhibit the human enzyme. This project focuses on the development of new inhibitors with improved potency and selectivity for the *M. tuberculosis* enzyme. Inhibitors containing a 5-phenyl group were targeted in order to increase lipophilicity and binding to the enzyme, whilst reducing binding to the human enzyme. Condensation of diethyl phenylmalonate with guanidine followed by chlorination and amination of the carbonyl group gave 2-amino-6-chloro-4-*p*-methoxybenzylamino-5-phenylpyrimidine, which was deprotected to give 6-chloro-2,4-diamino-5-phenylpyrimidine. Reaction of 2-amino-6-chloro-4-*p*-methoxybenzylamino-5-phenyl-pyrimdine with different aromatic and aliphatic amines was also investigated. Amination with an amino alcohol in presence of potassium carbonate in the absence of solvents was used to synthesise a number of analogues. Deprotection of the *p*-methoxybenzylamine was achieved by DDQ oxidation to give the desired 2,4-diamino-5-phenyl-6-aminoalcohol-pyrimidine products. The synthesis of the triol motif began with protection of ribonolactone and reduction of the carbonyl group to give the diol. Derivatisation of the product with various protecting goups was investigated.

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List of Abbreviations

AcpM	Acyl carrier protein
Ar	Aromatic
ATP	Adenosin triphosphate
BCG	Bacille Calmette-Guérin vaccine
Bn	Benzyl
BINAP	2,2'-Bis(diphenylphosphino)-1,1'-binaphthyl
br	Broad
^{13}C, C	Carbon
CAN	Ceric ammonium nitrate
CDC	Centers of disease control and prevention
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
$(CD_3)_2SO$	Dimethyl sulfoxide
CO_2	Carbon dioxide
CNS	Central nervous system
COSY	Correlation spectroscopy
d	Day, doublet
dd	double doublet
DCM	Dichloromethane
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMF	Dimethylformamide
DMP	Dimethoxypropane
DNA	Deoxyribonucleic acid
dTMP	Thymidine monophosphate
dUMP	Deoxyuridylate
DOT	Direct observed therapy
E. coli	*Escherichia coli*
EtOH	Ethanol

EtOAc	Ethyl acetate
GIT	Gastro-intestinal tract
Gln	Glutamine
GTP	Guanosine triphosphate
H, h	Hydrogen, hour
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
KasA	Ketoacyl ACP synthase
K_2CO_3	Potassium carbonate
LAM	Lipoarabinomannan
Leu	Leucine
$LiAlH_4$	Lithium aluminium hydride
MDR	Multi-drug resistant
MAC	*Mycobacterium avium complex*
M. africanum	*Mycobacterium africanum*
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
M. caprae	*Mycobacterium caprae*
M. chelonae	*Mycobacterium chelonae*
M. fortuitum	*Mycobacterium fortuitum*
M. tuberculosis	*Mycobacterium tuberculosis*
M. kansassi	*Mycobacterium kansassi*
M. leprae	*Mycobacterium leprae*
M. microti	*Mycobacterium microti*
M. ulcerans	*Mycobacterium ulcerans*
MeOH	Methanol
MOTT	*Mycobacteria* other than tubercle bacilli
m.p	Melting point
MS	Mass spectrometry
N	Nitrogen
$NaBH_4$	Sodium borohydride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH	Sodium hydride

$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
O, O_2	Oxygen
PAS	*p*-Aminosalicylic acid
PCL	*Pseudomonas cepacia* lipase
Pd	Palladium
$Pd_2(dpa)_3$	Tris(dibenzylideneacetone)dipalladium
$Pd(OAC)_2$	Palladium acetate
PG	Protecting group
Ph	Phenyl
PMB	4-methoxybenzyl
POC	Point of care
PPD	Purified protein derivative
i-Pr_2O	Diisopropyl ether
PZA	Pyrazonic acid
s	Singlet
S. aureus	*Staphlococcus aureus*
SHMT	Serine hydroxymethyl transferase
t	triplet
TBDPS	*tert*-butyldiphenylsilyl
TFA	Trifluroacetic acid
THF	Tetrahydrofuran, tetrahydrofolate
TLC	Thin layer chromatography
Trityl, Tr	Triphenylmethyl
Trp	Tryptophan
TS	Thymidylate synthase
WHO	World Health Organisation
XDR	Extensive drug resistance

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1. Introduction

1.1 The Genus Mycobacterium

The genus mycobacterium comes from the family *Mycobacteriacea*. The bacteria are thin, non-motile, and non-spore-forming, with a slightly curved shape and the genus contains more than 50 species.[1] They are gram positive bacteria[2] and can be found in water, soil, and food. Mycobacteria require long incubation times, specific media, and specific temperature to grow. They are also aerobic, and grow in environments containing 3-11 % CO_2.[1]

1.1.1 Classification of Mycobacterial species

Mycobacterial species are divided into two groups in terms of their growth: rapid, and slow growing. Some new species of both groups have been discovered in the last ten years.[1] Examples of fast growing bacteria are *M. chelonae* and *M. fortuitum,* both of which cause skin infections. An example of the slow growing bacteria is the *Mycobacterium avium complex* (MAC), a heterogeneous group of mycobacterial organisms that can affect the bronchi and lymph nodes, such as *M. kansassi* which causes pulmonary disease. *M. tuberculosis complex* is also slow-growing, and includes the specific species *M. tuberculosis, M. bovis, M. africanum, M. microti,* and *M. caprae. M. bovis* can be transmitted through unpasteurized milk.[1] Any of the *M. tuberculosis complex* microorganisms can cause tuberculosis in humans.[3] The slow growing microorganisms can be further subdivided into three classes. Photochromogenic, refers to organism's cells that produce pigments when exposed to light. In contrast, scotochromogenic organisms produce pigments constitutively even in the absence of light. Non-chromogenic organisms do not produce pigments at all. Organisms in the *M. tuberculosis complex* are non-chromogenic.[1]

Another method of classifying mycobacterial species is their pathogenicity, which is the ability of the organism to cause infectious disease. The first class is pathogenic mycobacteria, mainly *M. tuberculosis.* Mycobacteria other than *M. tuberculosis* are often referred to as MOTT, which stands for mycobacteria other than tubercle bacilli. MOTT are also called atypical mycobacteria, meaning they are not contagious. The

second class is the non-pathogenic mycobacteria. Many mycobacteria that are non-pathogenic are normal flora found in humans.[1]

1.1.2 The organism *M. tuberculosis*

One mycobacterial species, *M. tuberculosis,* is considered to be the causative pathogen of tuberculosis, which infects one–third of the world's population.[4] Robert Koch discovered that *M. tuberculosis* was the bacteria responsible for the disease tuberculosis in 1882. This disease is considered an epidemic, with around 9.8 million new victims a year.[5] For this reason, considerable efforts are focused on enhancing the treatment of tuberculosis. Because poor environmental conditions are considered to be the main cause for the spread and transmission of tuberculosis, improving these environments is also of high priority.[5]

M. tuberculosis grows in highly oxygenated environments,[6] which is why the disease often presents with pulmonary symptoms. The bacterium appears as a rod shape when examined by microscopy and is related to actinomycetes, which are a heterogeneous group of gram-positive bacteria with filamentous growth shapes.[7] *M. tuberculosis* is pathogenic with a slow generation time, between 15-20 hours.[6]

1.1.3 Morphology of *M. tuberculosis*

The cell wall structure of mycobacteria is unique among all eukaryotes and prokaryotes. It was first described by Minnikin in 1982.[8] The cell envelope consists of a cytoplasmic membrane comprising of a phospholipid bilayer, which is surrounded by a peptidoglycan cell wall.[9] The cytoplasmic membrane acts as a barrier.[2] The peptidoglycan layer is absent in plants, animals, and humans, unlike the bacteria, and it is thicker in gram positive bacteria than in gram negative.[10]

The cell wall of *M. tuberculosis* (Figure 1) also contains specialised very-long-chain fatty acids (60-90 carbon atoms) known as mycolic acids.[11] Mycolic acids contain number of unusual features and cyclopropane rings. It has been reported that the mycolic acid structure consists of α, β, keto methoxymycolates at the proximal and distal ends.[4] The exact structure of the mycolic acid layer has still not been completely

determined but it is known to play an essential role because any changes or mutations in the mycolic acid layer are fatal to the mycobacteria, as this changes the cell wall's permeability. The mycolic acid layer makes up about 30-40% of the cell wall.[1]

Linking the mycolic acid and peptidoglycan layers is the arabinogalactan layer,[2,11] also known as as the electron dense outer layer, electron translucent region,[9] or murein layer.[10] The layer comprises a linear polymer of galactose with branched arabinogalactan molecules.[2] Another layer of the cell wall structure of *M. tuberculosis* is the lipoarabinomannan (LAM) layer. The LAM layer which is a bulky polymer of arabinose and mannose intercalates the arabinogalactan layer (Figure 1).[12]

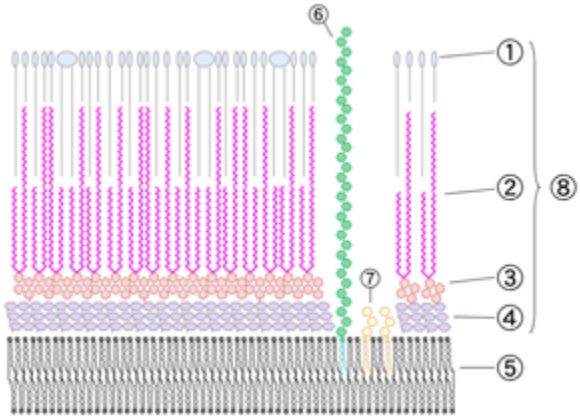


Figure 1: The cell wall structure of *M. tuberculosis.*

1- Outer lipids, 2- Mycolic acid, 3- Polysaccharides (arabinogalactan), 4- Peptidoglycan, 5- Plasma membrane, 6- Lipoarabinomannan (LAM), 7- Phosphatidylinositol mannoside, 8- Cell wall skeleton.

1.1.4 The effect of the cell wall structure of *M. tuberculosis* on its pathogenicity

Because of the lipid nature of the cell membrane, a substance has to dissolve in it in order to penetrate the membrane. Hydrophilic compounds generally require protein transporters to help them penetrate the lipid layers of the *M. tuberculosis* cell membrane. These transporters, such as the porin channels, are in the outer membrane of the mycobacteria. It has been suggested that these channels are connected to the inner layer of the cell membrane to facilitate the efflux of drugs and thus mediate resistance to antibiotics. Thus, they could be a treatment target for tuberculosis.[2]

Virulent strains of *M. tuberculosis* also possess cord factor, which is a surface glycolipid.[13] It is very toxic to mammalian cells, as it enhances acute and chronic tuberculosis and interferes with the action of isoniazid which is one of the first line drugs used to treat tuberculosis.[14] Some mycobacteria form structures such as capsules and biofilm, which are also associated with virulence. However, there is no exact information about their mechanism.[2]

The high lipid content in the cell wall, formed by the mycolic acid-arabinogalactan-peptidoglycan layers, gives mycobacteria many characteristics. For example the layer confers resistance to some anti-tuberculosis drugs.[2] The drug resistance is due to the high hydrophobicity, and hence acts as a permeability barrier. This hydrophobicity also makes the cell wall impermeable to acids (referred to as acid fastness)[9] and gives the mycobacteria the ability to survive inside macrophages.[6] The cell wall is also responsible for the immune response often observed following bacterial attack on the human body[9](Figure 1).

The presence of the cell wall allows identification of mycobacteria by acid-fast staining. The mycobacterial cells are treated with a red carbolfuchsin dye and then exposed to heat. Thereafter, the cells are treated with acid or alcohol to remove the red colour of the carbolfuchsin dye. These colourless cells are treated with methylene blue but the cells retain the red colour of the carbolfuchsin because the acid or alcohol does not wash off the initial red colour.[1]

1.2 Classification of the disease tuberculosis

Tuberculosis can be classified into three phases, according to the stage in the pathogenesis of the disease. First is the active or primary phase, which occurs upon infection. Second is the latent phase,[15] in which the disease becomes dormant. Third is the post-primary disease phase, which occurs when people who recover from the primary disease are re-exposed to mycobacteria.[16]

Another method of classifying the disease is based on the location of the infection. When the organism affects the lungs, it is called pulmonary tuberculosis, which is the most common type. If the organism causes lesions and shows up on the chest X-ray as

"millet seeds," this is called miliary tuberculosis. Extrapulmonary tuberculosis includes infection of any organ other than the lungs, such as the lymph nodes (also known as lymphanditis). Mycobacteria can also affect the central nervous system (CNS) and cause meningitis. Other organs that are less commonly affected by *M. tuberculosis* are the gastrointestinal tract (GIT), bones, and joints. Infection of the GIT, which may cause ascites (accumulation of fluids), was common in the past and occurred through ingestion of milk contaminated with *M. bovis*.[16]

1.2.1 Aetiology and transmission

There are many risk factors that increase the chance of contracting tuberculosis.[15,16] These factors include: close contact with infected people, poor nutrition, drug addiction; alcoholism and diabetes mellitus. In the last twenty-five years co-infection of HIV patients has also become an increasing problem. Other factors that make a tuberculosis epidemic more likely are poor living or working conditions and wars.[15]

1.2.2 Pathogenesis of tuberculosis

Tuberculosis can be transmitted by air by patients with an active pulmonary or laryngeal tuberculosis, cough, or sneeze, or even through talk. If bacilli are inhaled by another person, the inhaled bacilli can pass through the bronchi, reach the terminal alveoli in the lungs and cause active primary disease.[16] Inhaled droplet nuclei are 1-5 nm in diameter, and they are especially easily transmitted in small, crowded places that allow close contact. Extrapulmonary tuberculosis (an infection that occurs outside the lungs) accounts for a larger proportion of cases in countries in which the incidence of tuberculosis is relatively low, such as Western Europe and North America. However, more than 85% of tuberculosis cases worldwide are pulmonary.[15]

In the case of pulmonary tuberculosis, when the inhaled droplets reach the lungs, they are engulfed by macrophages. Inside the macrophages, the *M. tuberculosis* bacilli multiply and immune response components, such as cytokine and interferon, they are secreted in response. Usually the immune response occurs approximately two to eight weeks after the initial infection. There are two mechanisms for the macrophage immune response; first, the phagosome that contains the *M. tuberculosis* is infused with a

lysosome. This infusion causes bacterial lysis, forming toxic compounds. In the second mechanism, the ubiquitin peptide penetrates the mycobacterial membrane thus allowing access of toxic compounds into the permabilised cells. When the macrophages secrete immune components, the infection starts. However, these components cannot destroy the mycobacteria. The mycobacterial bacilli can avoid the fusion between the phagosome and the lysosome (first mechanism) and can escape from the macrophages with the help of some antigenic proteins that are secreted in the initial phase. However, other macrophages will engulf the escaped bacilli again to continue the immune response.[15]

A granuloma can also be formed as a result of the immune response. There are two types of granuloma; caseous granulomas consist of epithelial macrophages, neutrophils, and fibroblasts. In contrast, fibrotic granulomas consist of fibroblasts only. Mycobacterial cells can be present in three locations in these tuberculosis lesions. Most of the cells are extracellular, located in the wall of the cavity lesion, some of the cells are inside the caseous lesions; and others are inside the acid milieu of the macrophages.[6] The tubercule bacilli can also infect other organs, such as the central nervous system (CNS), kidneys, bones, and liver. Normally, development of a granuloma prevents mycobacteria from spreading to other sites in the body, and should lead to mycobacterial death. However, virulence factors in *M. tuberculosis* cells, such as the intracellular growth of mycobacteria in the macrophages, leads to ineffectiveness of the immune response. Mycobacteria can also resist radical reactive oxygen species produced during phagocytosis. The slow generation time of the mycobacteria also results in a delay in the immune system's ability to recognise the organism. Moreover, the presence of cord factor[15] results in death of macrophages and causes a caseating granuloma through interaction with lipids.[14] These factors reduce mycobacterial cell death and increase the persistence of the infection.

Because *M. tuberculosis* bacilli have the ability to form spores they are tolerant to low O_2 levels, low levels of nutrients, the pH in the alveoli and the toxic radicals secreted by macrophages by becoming dormant for years. This dormant state is called latent tuberculosis and generally has few symptoms. However, the disease can be re-activated by co-infection with other species (including HIV) and this can give rise to extra-pumonary tuberculosis.[15]

1.2.3 Diagnosis of tuberculosis

Early diagnosis increases the probability of successful treatment. The World Health Organisation (WHO) reported 63% of tuberculosis cases in 2009 because of delayed diagnosis. Delayed diagnosis is a common cause of tuberculosis becoming an epidemic, and this happens when the patient delays asking for medical help for any reason, especially if they have poor access to healthcare or diagnostic systems. Lack of reliable diagnostic tests or availability in many countries raises both the incidence and mortality rate of tuberculosis.[17]

The first diagnostic step is to perform microscopy on the patient's sputum, but more than one sample at different times is required because the long growth time of the mycobacteria means that a false negative result can be obtained. Tuberculosis can also be diagnosed using a chest X-ray since around 80% of cases are pulmonary. The disease appears as shadowing in the upper lobe or as fluids around the lungs.[11] Another method for diagnosis is the tuberculin test, which is is used for detecting the initial immune response and is most effective when performed six to eight weeks after the infection. The test requires intradermal injection of a purified protein derivative (PPD) from *M. tuberculosis*, which results in visible swelling of the skin due to an enhanced immune reaction.[16] Latent tuberculosis has few symptoms and generally requires the use of molecular biology techniques to determine the presence of bacterial genomic sequences.[15]

Some countries have a system of diagnosis called point of care (POC), which detects diseases such as HIV and hepatitis. These tests work by detecting the antibodies that have been produced by the human body as an immune response to infection. These tests have the advantage that they can be used in hospital or community settings without the need for laboratory facilities. In the case of *M. tuberculosis* infection there are some biological markers which are produced as part of the immune response. To detect these biological markers, there are some tests are called interferon-γ release assays, which detect patients with latent tuberculosis who were vaccinated by BCG. However, it is impossible to differentiate between the active and the latent stages of the disease. Also, there are some tests that detect antigens produced in response to *M. tuberculosis* infection, which can be used for blood or sputum samples. However, most of these

diagnostic tests have not been approved by WHO. There are ongoing studies to look for diagnostic tests that detect the metabolites from both the mycobacteria and the host.[17]

1.2.4 Epidemiology:

WHO has estimated tuberculosis incidence, prevalence, and mortality cases worldwide. It has been reported that 9.4 million new cases of infection and 1.78 million deaths are caused by tuberculosis every year. Of these cases, 14% are co-infected with HIV and 86% are patients infected by multi-drug resistant (MDR) strains.[22] The highest tuberculosis infection rates are found in Asia followed by Africa, and this is due to the spread of HIV in Africa. The highest proportion of multi-drug resistant cases is found in India and China.[18]

1.2.5 Signs and symptoms of tuberculosis

Primary tuberculosis patients are asymptomatic,[15] whilst pulmonary tuberculosis patients show many signs and symptoms. These include the coughing up of blood, weight loss, fever, fatigue, shortness of breath, and chest pain. Childhood tuberculosis can be asymptomatic and, in many cases, causes meningitis. Miliary tuberculosis, which occurs after the primary phase of disease, causes weight loss, anorexia, and fatigue.[16]

1.2.6 Prevention of tuberculosis

There are two types of tuberculosis preventions. A pharmacological intervention involves vaccinating healthy people against tuberculosis. The other intervention is a non-pharmacological intervention, which involves isolating the infected person in a separate room, thus preventing spread of the disease.[6]

The BCG (**Bacille Calmette–Guérin**) vaccine is currently used at birth. While the vaccine has shown to be effective for over ten- fifteen years, it does not prevent the adult tuberculosis disease. There is a need to develop a vaccine that can be more effective than BCG. A vaccine based on the heat shock protein HSP60 is now considered to prevent the reactivation of active tuberculosis.[6] This vaccine has some effectiveness against both pre- and post-exposure to mycobacteria.[19]

Vaccination is the best preventive method, especially for latent tuberculosis. There are more than ten vaccines in clinical trials, two of which are recombinant *M. bovis* BCG. Some of these vaccines are also used as booster vaccines to control the immune response after exposure to BCG. Studies on vaccines to prevent the reactivation of tuberculosis are ongoing, continuing the search for a good replacement for BCG.[15]

The studies to find a BCG replacement are focused mainly on the immune cells. Many studies have shown that prevention mainly depends on the immune cells especially the T-cells and the interleukin because of their ability to produce toxins that attack bacteria. These studies are focused on enhancing protection against the first exposure to bacteria, which prevents the reactivation of the latent tuberculosis and also lowers the chance of latent tuberculosis development.[20]

1.2.7 Treatment of tuberculosis

Before the 20th century, the only available treatment of tuberculosis was bed rest, good nutrition and a healthy environment.[6] At the beginning of the 20th century, the cell wall structure of *M. tuberculosis* became the main target of study, especially after penicillin and sulphonamides had been shown to be ineffective at treating tuberculosis.[21] During the 1940s, the first drugs for tuberculosis treatment were introduced, streptomycin and *p*-aminosalicylic acid. Isoniazid was then released and became the most effective anti-tuberculosis drug. Ethambutol was introduced in the 1960s to replace *p*-aminosalicylic acid because of its side effects. Therapy usually required eighteen to twenty four months of treatment. However, upon the introduction of rifampicin as a treatment in the 1970s, the standard course of treatment was reduced to six to nine months.[6]

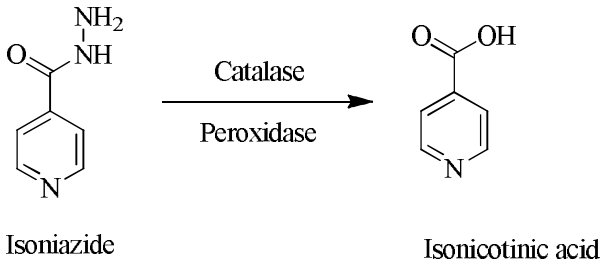
1.2.8 First line drugs

The first line treatment of tuberculosis uses a combination therapy in which isoniazid, rifampicin, streptomycin, and pyrazinamide are administered at the same time. Each of these drugs in this regimen acts on a different bacterial target and has a specific mechanism of action. This initial treatment usually lasts for two months, during which time the patient has daily administration of the four drugs. Following completion of this

course of treatment, the patient receives isoniazid and rifampicin three times a week for the next four months. [21]

1.2.8.1 Isoniazid

Isoniazid is a prodrug that is activated by a peroxidase enzyme to produce isonicotinic acid (Scheme 1). Isoniazid inhibits mycolic acid synthesis by inhibition of two enzymes: inhA enzyme, and a complex of an acyl carrier protein (AcpM) and β-ketoacyl synthase (KasA) enzymes. InhA, AcpM and KasA enzymes are required for the activation of isoniazid prodrug and they are also involved in the synthesis of mycolic acid.[22] Isoniazid is highly active against rapidly growing bacteria.[6] Isoniazid has a bacteriostatic effect against inactive mycobacteria. It also has an effect on DNA and carbohydrates by interfering with multiple enzymes that are responsible for DNA metabolism.[21] It is the most powerful drug to treat tuberculosis, but it has a few uncommon side effects such as insomnia, anxiety, and an influenza-like syndrome.[16]



Scheme 1: Activation of isoniazide by catalase peroxidase enzyme.

1.2.8.2. Rifampicin:

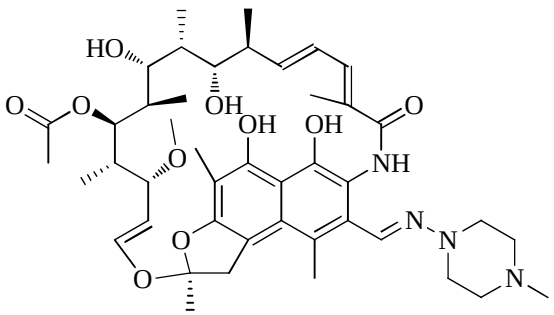
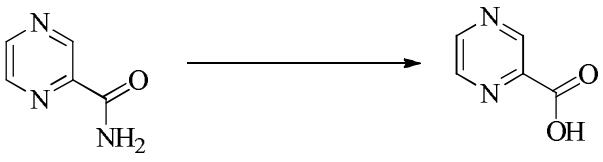


Figure 2: The structure of rifampicin.

Rifampicin is a broad spectrum bacteriostatic antibiotic. It disrupts the nucleic acid biosynthesis by inhibiting the activity of DNA-dependent RNA polymerase.[22] Rifampicin is the second most powerful anti-tuberculosis drug. It has a bactericidal effect against the intracellular *M. tuberculosis* bacilli. It has some effect at both the initial infection stage, as well as the later stages of tuberculosis. Rifampicin has some side effects, such as thrombocytopenia, skin reactions, and an influenza-like syndrome. Rifampicin is metabolised by hepatic cytochrome P_{450} enzymes and drug-drug interactions are very common, especially when co-administered with corticosteroid and anticoagulant drugs.[16]

1.2.8.3 Pyrazinamide

Pyrazinamide is used for short-term therapy of tuberculosis. It has a bacteriostatic effect against inactive mycobacteria, mediated by disruption of the mycobacterial cell membrane.[6] Pyrazinamide is a prodrug that is converted to the active form pyrazonic acid (PZO) by pyrazinamidase (Scheme 2). Pyrazonic acid is not readily cleared by the antibiotic efflux system and accumulates in the bacterial cytoplasm, leading to a reduced pH and inhibition of fatty acid synthase, which is required for fatty acid biosynthesis.[22] Pyrazinamide has some unwanted side-effects such as hepatotoxicity, anorexia, and photosensitisation.[16]



Pyrazinamide Pyrazonic acid

Scheme 2: Activation of pyrazinamide to pyrazonic acid by pyrazinamidase.

1.2.8.4 Ethambutol

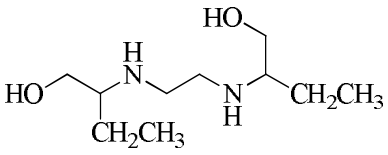


Figure 3: The structure of ethambutol.

Ethambutol is an inhibitor of the arabinosyltransferase enzyme,[21] which affects the polysaccharide layers and inhibits the conversion of *D*-glucose to *D*-arabinose residues. As a result, synthesis of the arabinogalactan layer of the mycobacterial cell wall is disrupted.[6] Ethambutol therefore has a bacteriostatic effect.[23] The main side effect of ethambutol is optic neuritis, which causes visual changes and often goes unnoticed by children (unless children and parents are counselled to watch out for such changes). Other side effects of ethambutol include jaundice and skin rash.[16]

1.2.8.5. Streptomycin:

Streptomycin is transferred to the bacterial cells by active transport. It disrupts the transcription of the genetic code, and as a result, it inhibits the protein synthesis of the mycobacterial cell wall.[6] Streptomycin has some unwanted side effects, such as allergic reactions, renal impairment, and dermatitis.[16]

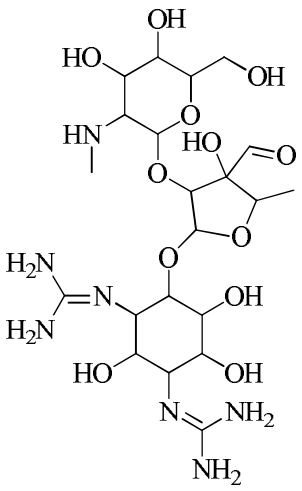


Figure 4: The structure of streptomycin.

1.2.9 Second line drugs

There are a number of second line drugs that can be used for treatment of tuberculosis, but these antibiotics have many side effects, are expensive, and are less selective against *M. tuberculosis*. Second line antibiotics include capreomycin, cycloserine, amikacin, ciprofloxacin, and fluoroquinolones.[2] Because of the unwanted side-effects of second line drugs, they are primarily used in the treatment of resistant bacteria.[6]

1.2.10 Multi-drug resistance (MDR)

Microbial antibiotic resistance occurs when a previously effective drug treatment becomes less effective. It differs from the phenomenon of insensitivity, in which the antibiotic is always ineffective. Infections caused by resistant micro-organisms respond less well to treatment and they cause increased mobidity and mortality. The first report of drug resistant tuberculosis was in the 1940s.[4] A further complication is that some strains of mycobacteria are naturally resistant to at least one of the first line drugs used for treatment of tuberculosis; thus, new treatments should begin with a combination of drugs to maximise effectiveness and minimize the emergence of new resistant strains. Mycobacterial resistance can occur simultaneously against isoniazid and rifampicin, the most powerful anti-tuberculosis drugs. The WHO calls this multi-drug resistant tuberculosis.[6]

There are many risk factors that increase the possibility of multi-drug resistance. These include: long-term administration of anti-tuberculosis drugs, close contact with other patients infected with multi-drug resistant strains, patients who are immuno-compromised, such as those co-infected with HIV, drug and alcohol addiction, immigration from areas in which tuberculosis is common and homelessness.[6]

1.2.10.1 Classification of drug resistance

Drug resistance is classified by the WHO into four groups:

- Mono-resistance, in which tuberculosis strains are resistant to a single drug (usually isoniazid or streptomycin).
- Double resistance, in which strains are resistant to both isoniazid and streptomycin.
- Triple resistance, in which strains are resistant to isoniazid, streptomycin, and rifampicin.
- Quadruple resistance, which occurs when strains are resistant to isoniazid, streptomycin, rifampicin, and ethambutol.[6]

1.2.10.2 Types of multi drug resistance

There are two main sub-types of multi-drug resistance. Primary resistance occurs when patients are infected by tuberculosis strains which are already resistant. Acquired resistance is where the patient is infected by non-resistant strains but resistance subsequently develops. Most of the cases of multi-drug resistance are acquired,[24] and this is partly a consequence of the long treatment times which are required due to the slow growth of the mycobacteria.

1.2.10.3 Extensive drug resistance (XDR)

Extensive drug resistance (XDR) is a situation in which mycobacterial cells become resistant to the second line drugs, especially to fluoroquinolones,[25] in addition to their resistsance to the first line drugs.[5] In 2008 the WHO reported that 5.4% of multi-drug resistant strains had extensive resistance, with eight cases reported in the U.K. In 2010 there were fifty three thousand reported cases of multi-drug resistance, of which sixty were reported in the U.K.[26] MOTTs can also become resistant to anti-tuberculosis drugs, especially *M. ulcerans*.[1]

1.2.10.4 Mechanism of resistance

The specific mechanism of resistance differs depending on which specific antibiotic is used. Generally any non-lethal mutation or change in any gene of mycobacterial cells will result in resistance if this affects the antibiotic function of the drug. For example, isoniazid is activated by catalase (specific type of peroxidase enzyme) to create isonicotinic acid (Scheme 1), any mutation that reduces the activity of this enzyme will lead to inactivation of the isoniazid since the pro-drug is less efficiently converted into the active antibiotic. Mycobacterial resistance to isoniazid can also result from mutations in a protein called Inh-A, which is responsible for mycolic acid synthesis.[6]

The mechanism of rifampicin resistance is related to mutations of RNA polymerase, which is responsible for nucleic acid synthesis. Likewise, streptomycin resistance arises due to mutation of ribosomal RNA genes[6] or the transporters which are responsible for the active transport of streptomycin through the bacterial cells.[16]

Isoniazid and rifampicin are effective against the extracellular bacilli, often located inside caseous lesions and macrophages, while pyrazinamide is effective against the intracellular cells where the environment is acidic. In the latent stage, isoniazid, rifampicin, and pyrazinamide fight bacteria slowly. Discontinuing the therapy prematurely is likely to cause resistance[6] due to incomplete eradication of the mycobacteria. Multi-drug resistance can also occur due due to a combination of drugs.[23]

1.2.10.5 Treatments of tuberculosis multi-drug resistance

Treatment of tuberculosis with first line drugs regimen is effective in up to 95% of tuberculosis patients. In cases of multi-drug resistance development, treatment with second line drugs with DOT (Direct Observed Therapy) is effective in 50-70% cases. Treatment of extensive drug resistance is difficult because the standard first and second line drugs are less effective. The patient is usually treated with a combination of eight to ten drugs for twenty four months with DOT, but highly toxic side effects, such as nephrotoxicity and hepatotoxicity are often observed.[5] Other factors that need to be considered are:

- The risk of the patient developing MDR.
- The expense of the therapy.

- The likelihood of the disease been transmitted to others.[27]

Direct observed therapy (DOT) is required for patients who are at high risk of developing MDR, such as those co-infected with HIV. Patients who are not able to complete the treatment or have to be treated intermittently also require the use of direct observed therapy.[6]

Patients who are suspected of having MDR infections should not have further drugs added to their existing regimen. Instead the existing drugs should be replaced by the injectable second line drugs and treatment should be continued for at least twenty four months. In addition patients should undergo direct observed therapy to ensure better compliance and the infection tested for susceptibility.[6]

There are approximately twenty second line drugs for the treatment of tuberculosis. These include; injectable drugs, such as fluoroquinolones, which are effective in treatment of MDR, ethionamide, which inhibits mycolic acid synthesis, cycloserine, which inhibits peptidoglycan and arabinogalactan synthesis and amikacin, which, like rifampicin, inhibits bacterial RNA synthesis. The problem with these drugs is that they are less effective in the treatment of latent tuberculosis. Patients co-infected with HIV have the additional complication that the drugs used to treat this infection can interact with those used to treat tuberculosis. For example, protease inhibitors used to treat HIV often interact with rifampicin.[28]

1.2.11 Classifications of the anti-mycobacterial compounds regarding their mechanism of action

The anti-mycobacterials can be subdivided according to their mechanism of action. For example, isoniazid, pyrazinamides, pyrazoles, and nitroimidazopyrans all inhibit fatty acid biosynthesis. Cycloserine (Figure 5), ethylenediamines, and arabinose analogues all affect arabinogalactan or peptidoglycan biosynthesis and thus compromise the bacterial cell wall. The aminoglycosides, cyclic peptides, and macrolides inhibit protein biosynthesis, whilst rifampicin, fluoroquinolones, purine, and pyrimidine analogues inhibit nucleic acid biosynthesis and the deazapteridines and benzimidazoles which interfere with tubulin polymerisation and therefore inhibit cell division. Salicylamide and its analogues interfere with the biosynthesis of siderophores, which are required for

uptake of iron into the mycobacterial cells. Azoles inhibit the action of cytochrome P_{450} enzymes. Branched chain amino acid biosynthesis is inhibited by sulfometuron methyl, whilst signal transduction is inhibited by the salicylanilides, benzothiophenes, and nitropyrroles. There are also some miscellaneous inhibitors such as those which inhibit isocitrate lyase (an essential enzyme required for the bacterial glyoxylate cycle), the peptide deformylase inhibitors, and the pyridazinoindoles which inhibit monoamine oxidase. Finally there are the diaminopyrimidines, triazines, and deazapteridines, which interfere with folate metabolism by inhibiting dihydrofolate reductase (DHFR), the drug target examined in this project.[21]

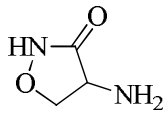


Figure 5: The structure of cycloserine.

Finding an efficient treatment for multi-drug resistance is difficult because of mycobacteria's long generation time, latent stage, and low metabolic activity. Because the location of the bacteria is inside the human body (*e.g.* in the pulmonary cavity or inside the lesions), this makes the delivery of the antibiotic to the bacteria difficult.[21]

1.2.12 Other treatment regimens and new antimycobacterial agents for tuberculosis

DOT is useful with patients who cannot complete the course of treatment or those who have intermittent treatment, such as that in the Denver regimen. The Denver regimen can be applied in two ways. In the first way, the first line drugs are administered daily for two weeks. The frequency of administration is then decreased to twice a week for another six weeks, followed by administration of isoniazid and rifampicin only twice a week for another sixteen weeks. The second method involves administration of the first line drugs three times a week for eight weeks, then the same dosage of isoniazid and rifampicin for eighteen weeks.[27] For both ways, DOT should be accompanied with drug toxicity monitoring.[6]

There are other regimens that have been investigated on latent tuberculosis patients that are currently under study. For example, one regimen studied in the UK involves the administration of isoniazid and rifampicin daily or twice a week for three months.

Another regimen that is under investigation is administration of isoniazid and rifapentine (a rifampicin analogue) once a week accompanied by DOT. The Centre for Disease Control (CDC) has suggested another treatment strategy. This treatment consists of six to twelve months of fluoroquinolones or ethambutol administered with pyrazinamide. Unfortunately, this regimen appears to be less effective.[29]

A more promising investigation involved rifapentine (Figure 6), a cyclopentyl rifampicin derivative used in a trial study on tuberculosis patients who were HIV negative. The administration of rifapentine combined with isoniazid once a week showed some effectiveness in these patients, but was not effective in HIV positive patients.[29]

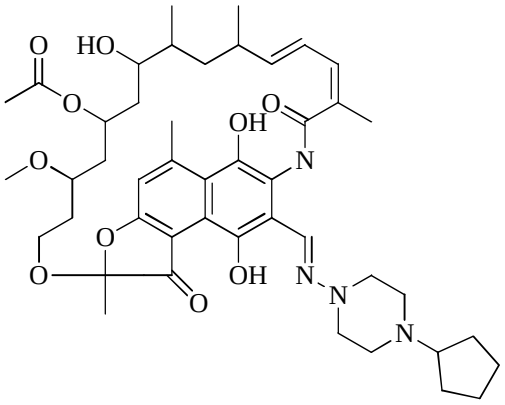


Figure 6: The structure of rifapentine.

There are anti tuberculosis approved drugs, such as imipenen, which are effective against both multi and extensive drug resistance, however, there still is not enough information about its effectiveness in humans (Figure 7).[29]

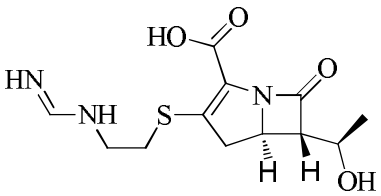


Figure 7: The structure of imipenem.

Another drug called meropenem,[29] which is a carbapenem, shows some activity against *M. tuberculosis* strains when it is combined with a β-lactamase inhibitor (Figure 8). Additionally, thioridazine has shown some activity in murine models against tuberculosis and multi-drug resistance strains through two interesting functions. It

inhibits bacterial efflux and ion transport in macrophages, allowing destruction of bacterial cells.[29]

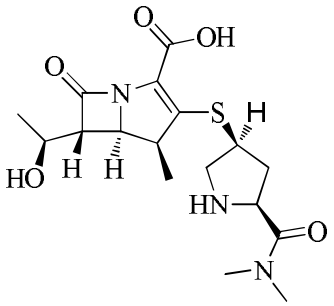


Figure 8: The structure of meropenem.

It has been reported that nine months of isoniazid treatment was curative for up to 90% of latent tuberculosis patients, but close observation of liver function and side effects is required. It has been reported that the administration of rifampicin alone for nine months showed lower hepatotoxixity.[30]

To find appropriate regimens to treat latent tuberculosis, animal models have been used to investigate the effectiveness of various drugs. For instance, the administration of rifapentine and moxifloxacin once weekly for three months was shown to be as effective as nine months of daily isoniazid. Another example of an animal tested drug is diarylquinolone, which proved to be better than isoniazid during the early stage of tuberculosis and better than rifampicin in the latent stage. Also 1,2-ethylendiamine was shown to be effective against tuberculosis in animal models. Scientists expect that these drugs will be used for the treatment of latent tuberculosis in the future.[15]

The search for an appropriate treatment of latent tuberculosis is still ongoing. Examination of tuberculosis shows that a functional glyoxylate shunt is important for maintaining the mycobacteria in a dormant state. The glyoxylate shunt is a modification of the tricarboxylic acid cycle in which the oxidative steps are bypassed, allowing the mycobacteria to utilise alternative carbon sources. The two key enzymes in the glyoxylate shunt are isocitrate lyase and malate synthase. Inhibitors of these two key enzymes have been shown to be effective for the treatment of latent tuberculosis.[4]

Multi-drug resistance is another problem that studies have focused upon. Fluoroquinolones are bacteriocidal, and are recommended for treatment of multi-drug resistance because of their bioavailability. Moreover, fluoroquinolones have no cross-

resistance with other resistant drugs.[29] Molecular techniques can be used to characterise resistant strains to determine the specific mutations present in a particular strain.[24]

1.2.13 Tuberculosis studies:

1.2.13.1 Studies based on screening techniques

Some of the new studies that target tuberculosis are focused on the genome sequencing of *M. tuberculosis*, and these kind of studies have still not shown any large effect.[5] One of these studies was based on screening enzymes and identifying their inhibitors. This method was useful for targeting mycobacterial growth; however, this method did not help in determining the biological target. The same study investigated a phenotypic screening of the bacterial cell, which gives a picture of the interaction between drug targets and any constituents of the bacterial cell. This kind of screening has led to a discovery of new anti tuberculosis drug candidates, such as diarylquinolines (TMC 207), which affect ATP synthesis, and benzothiazines (BTZ043), which affect arabinan synthesis (Figure 9).[5]

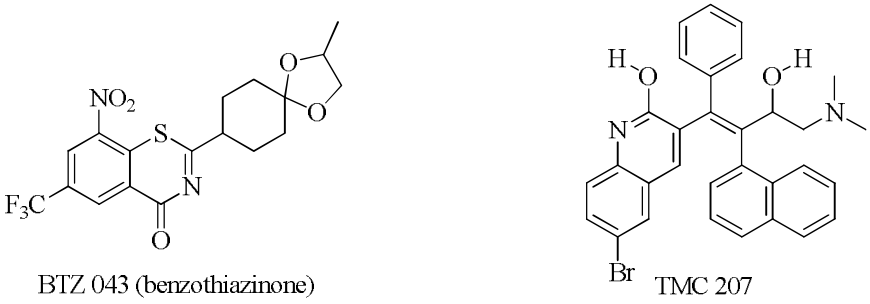


Figure 9: New anti tuberculosis candidates discovered by screening based techniques.

1.2.13.2 Reengineering of existing scaffolds

Kaul *et al*.[5] reported another approach for looking for new tuberculosis drugs, which are generated from existing antibiotics. These studies require reengineering of existing scaffolds, which means a modification is made to existing anti-mycobacterials to enhance their activity and other properties to achieve a better outcome. For example, oxazolidinones (linezolid) are anti-mycobacterial drugs that have been modified to give a better activity against *M. tuberculosis* and lower toxicity (Figure 10).[5]

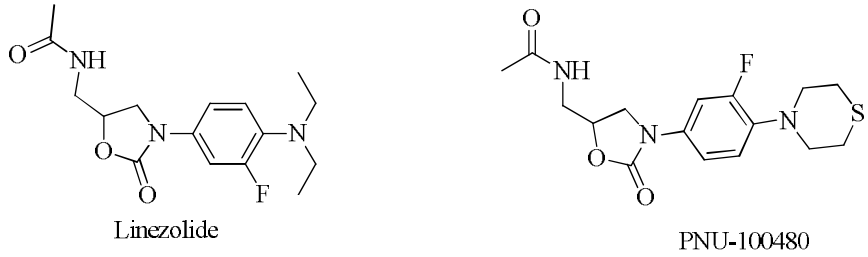


Figure 10: Modification of linezolide to form PNU-100480.

Another example are the nitroimidazole anti-folates, which are effective against anaerobic bacteria and parasites; however, they are still in clinical studies to test their affect on latent tuberculosis after their modification (Figure 11).[5]

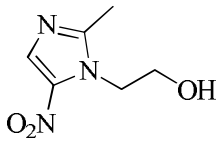


Figure 11: 1-Hydroxyethyl-2-methyl-5-nitroimidazole (Metronidazole).

1.2.13.3 Nanotechnology-based techniques

Tuberculosis studies have progressed to the application of nanotechnology for improving the drug delivery system. Nanoparticles give more effective and compliant therapy. A number of nanotechnologies have been reported, such as nanosuspensions (dispersions of pure nanosized particles) which enhance drug solubility. Nanosuspensions have been tested *in vivo* and have given interesting results in the development of new anti-tuberculosis drug formulations.[25]

Another nanotechnology that has been investigated is the use of nanoemulsions, the oil in water-based dispersion of drug particles. They are easy to prepare on a large scale and deliver.[25]

Niosome is a technique that allows flexibility in structure characterization of the drug. Polymeric nanoparticles also have been investigated and have shown improved stability of both hydrophilic and hydrophobic drugs.[25]

Modifying the surface of anti-tuberculosis drugs to make them more hydrophilic is a process called encapsulation. This technique will protect the anti-tuberculosis drugs from the immune response of infected patients. When applied to isoniazid and rifampicin, this technique led to higher intracellular bioavailability. Liposome or microsphere encapsulated anti-tuberculosis drugs are more effective than free drugs. Liposomes, nano vesicles that surround the encapsulation core of the drugs, will increase the length of release of the drugs.[25]

1.2.13.4 Tuberculosis studies on HIV patients

Many studies have focused on HIV patients who have tuberculosis. These patients are suffering from drug interactions between the antimycobacterial drugs and the anti-retrovirals, which lead to toxicity and immune reconstitution inflammatory syndrome. Therefore, studies have focused on the amount of time of using these anti-retroviral drugs. If the administration of anti-retrovirals is delayed until completion of the tuberculosis treatment course, this would cause death due to HIV progression. To overcome this problem, WHO recommended that treatment with anti-retrovirals should start within two to eight weeks after a patient begins a regimen of anti-tuberculosis drugs.[31]

1.2.13.5 Inhaled therapy for tuberculosis

Inhaled therapy for tuberculosis has been investigated. Inhaled therapy targets the lungs, the macrophages, and the dendritic cells that are produced by the immune system. This form of therapy reduces the side effects of anti tuberculosis drugs. These studies are still ongoing, though aerosol BCG vaccine has been tested on animals and has shown good activity.[32] Inhaled nanoparticles for the treatment of pulmonary tuberculosis are also beneficial as a local drug delivery system with low systemic side effects.[25]

1.2.13.6 Studies on second line drugs for tuberculosis

One type of second line drugs are quinoxalines, which are interesting antibacterial drugs. Moreno *et al.*[33] and his group synthesized many quinoxaline-1,4-di-N-oxide derivatives that they had tested on specific strains of *M. tuberculosis*. Primary screenings showed some activity. Moreno *et al.* and his team have reported some considerations for the development of quinoxaline-1,4-di-N-oxide derivatives as anti-mycobacterials. For instance, the quinoxaline ring with carboxamide at position 2, a methyl motif at position 3, and an electron withdrawing group at position 6 or 7 or both, they are all attached to an aromatic ring via a linker, such as a methylene group (Figure 12).[33]

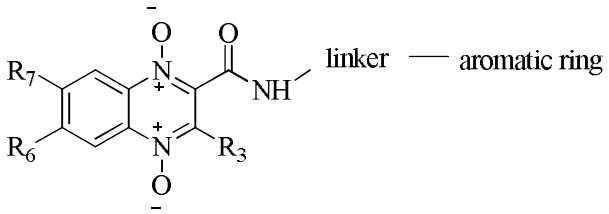


Figure 12: Design of quinoxaline-2-carboxamide-1,4-di-N-oxide.

Shunduru *et al.*[34] reported a synthesis of 2,4,6-trisubstituted-1,3,5-triazines derivatives, which was started from the previous reports about the pyrimidino-triazines compounds that have anti-leishmanial activity with moderate antibacterial activity (Figure 13). Studies found that 1,2,3 triazine moiety is important for anti-tuberculosis activity, especially when the drug is coadministered with isoniazid.[34]

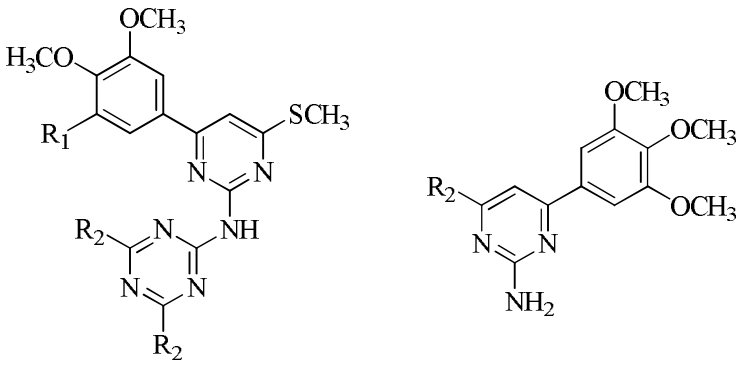


Figure 13: Pyrimidino-triazines and pyrimidines with anti leishmanial and anti tubercular activity.

Rajni *et al.*[35] reported a study that focused on the cell wall structure of mycobacteria, virulence factors, and the pathogenesis of the initial and the latent stages of the disease. They have reported that guanosine triphosphatase was a drug target for tuberculosis. The functional enzyme is required for the persistence of the bacteria and allows the mycobacteria to escape from the lysosome. They suggested that drugs targeting the GTP enzyme could provide novel treatments for latent tuberculosis.[35]

1.2.14 Dihydrofolate reductase (DHFR)

Prokaryotic and eukaryotic cells need folate to synthesise many cellular components.[36] Metabolic processes requiring folate include synthesis of nucleic acids and the regeneration of methionine. Oxidation and reduction of a one-carbon unit is required during these processes. Prokaryotes can synthesise folate while eukaryotes obtain folate from food or as supplement. The structure of folic acid consists of 2-amino-4-oxopteridine, *p*-aminobenzoic acid and glutamic acid.[36](Figure 14).

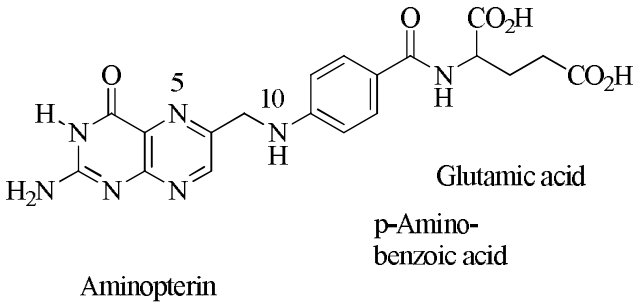


Figure 14: Structure of folic acid.

1.2.14.1 Folate cycle

Folate metabolism involves several reactions that modify the carbon atom that attaches to nitrogen atom N^5, N^{10}, or both of the folate structure (Figure 14).[37] The most important enzyme in folate metabolism is dihydrofolate reductase (DHFR), which reduces dihydrofolate to tetrahydrofolate using NADPH. The NADPH transfers a hydride anion to C^6 of the pteridine ring,[40] with concurrent protonation at N^5 position[41] (Scheme 3). The latter is required for biosynthesis of thymidylate and purines used for DNA and RNA synthesis, methionine, and several amino-acids.[38] Tetrahydrofolate is a carrier of a one-carbon unit, and it is formed by a reduction of 7, 8-dihydrofolate by DHFR. THF is converted to 5,10 methylene-THF by the interconversion of serine to glycine, which is catalyzed by serine hydroxymethyltransferase (SHMT).[37] 5,10-methylene-THF is then converted to dihydrofolate during thymidine synthesis. Deoxythymidylate (dTMP) is formed from deoxyuridylate (dUMP) by thymidylate synthetase (TS) using 5,10-methylenetetrahydrofolate as the source of the methyl group. The 7,8-dihydrofolate produced during this reaction is reduced to tetrahydrofolate by DHFR, thus allowing regeneration of the cofactor. So, TS and DHFR perform important steps in the synthesis of dTMP and DNA.[39]

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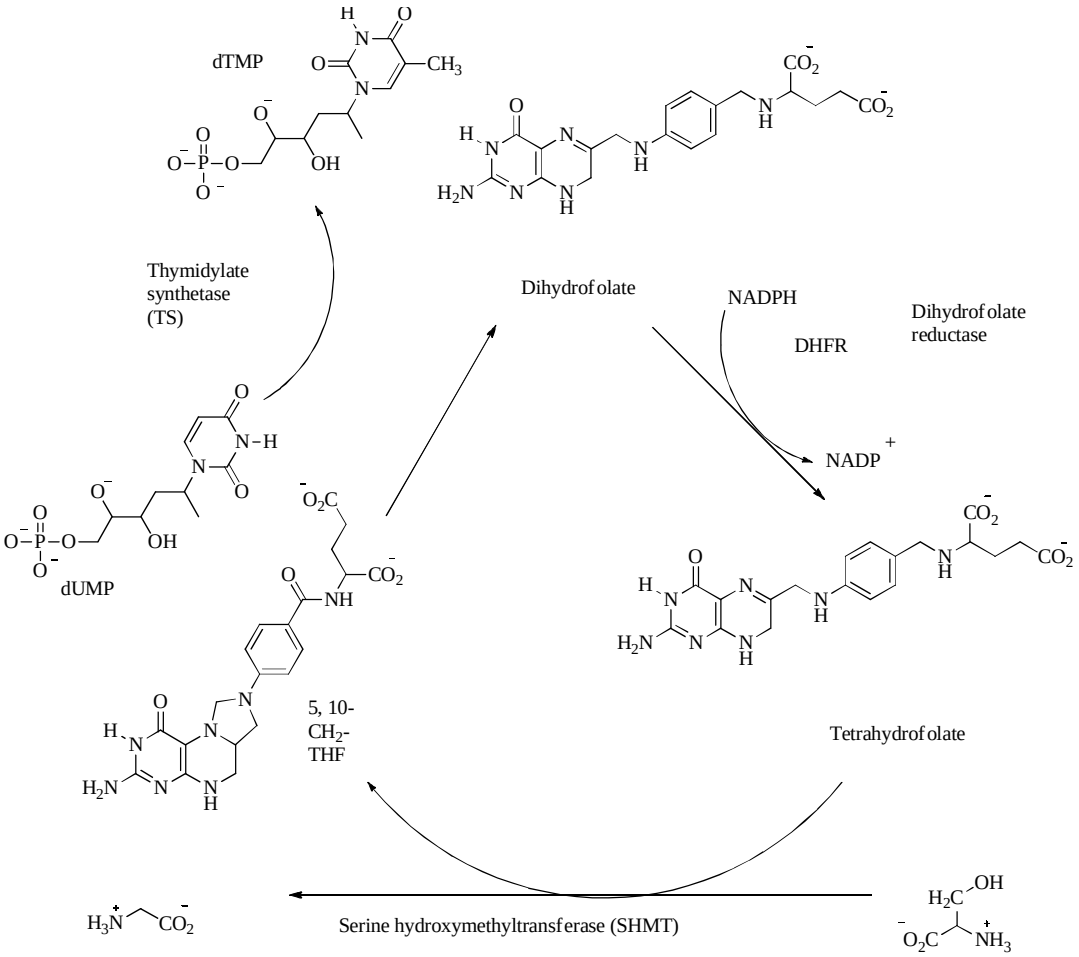
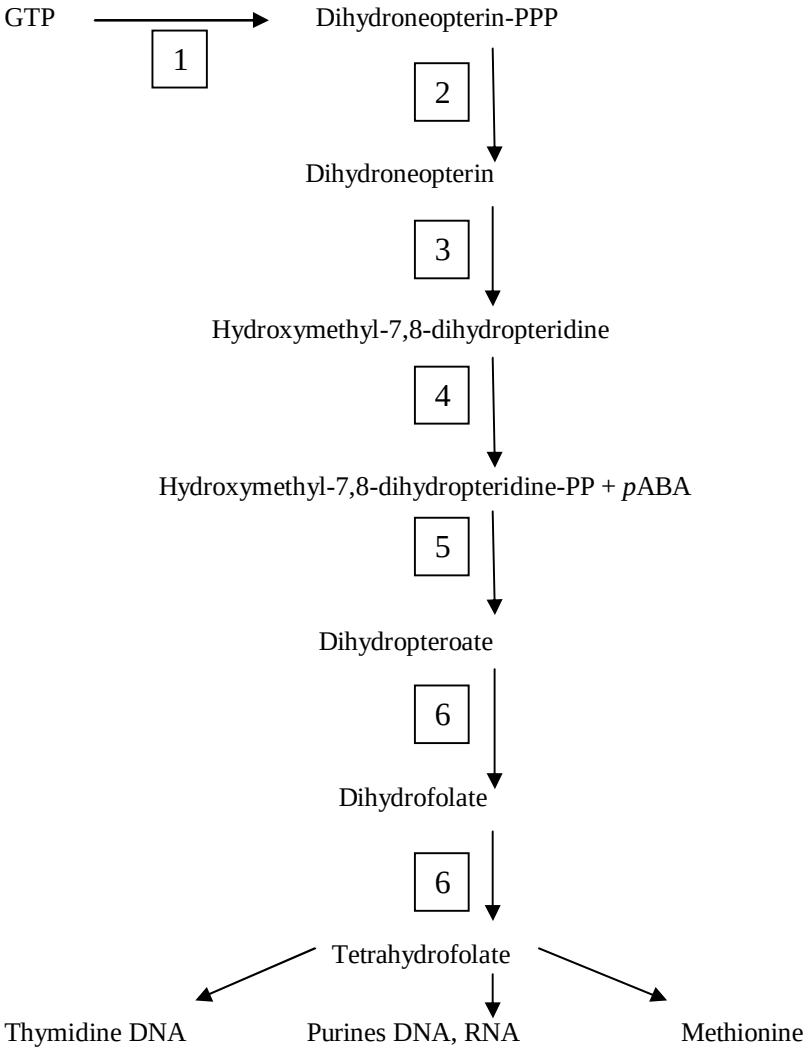


Scheme 3 : Biosynthesis of THF, DHF and 5,10-methylene THF in folate metabolism.

Six enzymes are important for folate metabolism; the scheme below shows the involvement of these enzymes (Scheme 4).[42]



Scheme 4 : Enzymes involved in folate cycle.

1-GTP cyclohydrolase; 2- Dihydroneopterin aldolase; 3- 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase; 4- Dihydropteroate synthase; 5- Dihydrofolate synthase; 6- Dihydrofolate reductase.[42]

1.2.14.2 *M. tuberculosis* DHFR three-dimensional structure

DHFR structure of *M. tuberculosis* consists of a general fold of central β-sheets with four α-helices. The central β-sheet consists of seven parallel strands and a single C-terminal strand, which is an anti-parallel strand. The coenzyme NADPH is bound in an extended conformation by the C-terminal strand of the β-sheet, while the nicotinamide ring is inserted between strands β-a and β-f.[38]

1.2.14.3 DHFR inhibitors

There are many DHFR inhibitors that have been studied[43] for selectivity and potency against many bacterial and protozoal diseases. Because of the role of DHFR in DNA and RNA biosynthesis, these drugs are also used for treating cancer. The most well-known inhibitor that binds tightly to DHFR is methotrexate (MTX) (Figure 15), which is a 2,4-diaminopyrimidine derivative. The structure below shows that methotrexate is an analogue of the dihydrofolate structure. The pteridine moiety contains an amino group instead of the carbonyl and the nitrogen atom of the *p*-aminobenzoic acid moiety has been methylated, which makes it a strong inhibitor.[36]

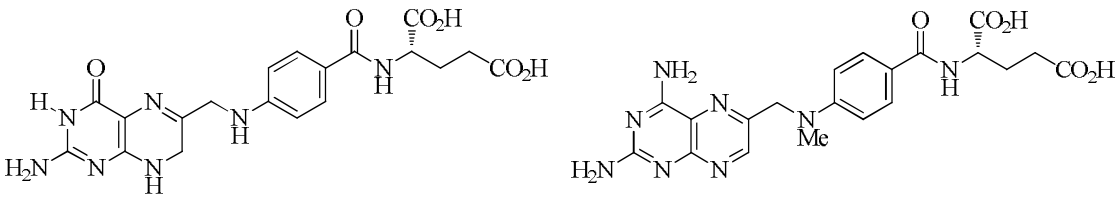


Figure 15: Structures of dihydrofolate (DHF) and methotrexate (MTX).

The conversion of the 4-keto group in dihydrofolate to 4-NH_2 group makes it a strong inhibitor because the amino group will form hydrogen bonds with the amino acids in the binding site of *M. tuberculosis* DHFR (Figure 15). Crystallographic studies have shown in very high resolution that methotrexate is in the protonated state in the binding site. Replacement of the NH_2 group at position 4 with SH, OH or CH_3 results in weaker inhibitors because hydrogen bonding with the amino acids in the binding active site of *M. tuberculosis* DHFR is lost.[44]

Methotrexate is also used as an anticancer drug. It is very potent against human DHFR, but because of its polar glutamic acid side-chain it needs active transport into cells.[43] Other examples of anti-folate drugs are trimethoprim and pyrimethamine (Figure 16). These are very effective drugs for the treatment of bacterial and protozoal infections.[45] There are also a number of very potent inhibitors of bacterial and protozoal enzymes, such as trimetrexate and piritrex,[38] but these drugs have serious side-effects. Comparison of the human and *M. tuberculosis* DHFR structures can be used to optimise selectivity of an inhibitor for the mycobacterial enzyme and thereby decrease the possibility of serious side-effects.

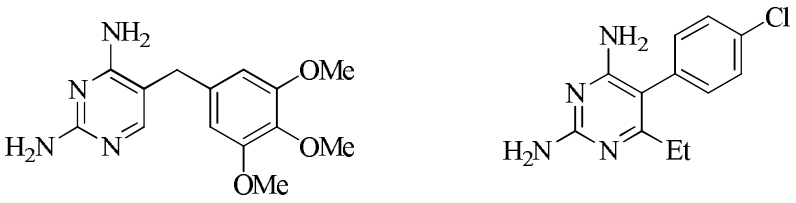


Figure 16: 2,4-Diaminopyrimidine derivatives.

1.2.14.4 DHFR inhibitor studies

Before 1930, the idea of the selectivity of chemotherapeutic treatment was not common because of lack of information about the detailed biochemical pathways in both eukaryotic and prokaryotic cells. The field was totally changed by the discovery of sulfonamide and its mechanism of action as an antibacterial. Sulfonamides compete with *p*-aminobenzoic acid in the synthesis of folic acid and this leads to the inhibition of cell growth due to the reduced availability of folate.[41]

In 1948, Hitching and his team discovered 2,4-diaminopyrimidines.[46] This discovery was followed by many studies centred around the development of drugs, including the nature of the enzymes or receptors that are involved are drug targets. The goal of these studies was to enhance the potency and selectivity of the 2,4-diaminopyrimidines. These studies led to the discovery of different classes of anti-folates with different selectivity and a broad spectrum. The studies also investigated the factors that increase the binding affinity to the enzymes from different species. [41]

DHFR inhibitors are used to treat many human diseases, including fungal infections, psoriasis, rheumatoid arthritis, and cancer. DHFR has become the focus of chemical and molecular biological studies because of its utility as a drug target. However, some cells do not respond to DHFR inhibitors because they do not synthesize thymidylate and DNA, which are the main pathways requiring folate. [41]

1.2.14.5 Bacterial DHFR

Folates are produced by *de novo* synthesis in bacteria and plants. Kompis *et al.*[45] reviewed the DHFR inhibitor literature between 1995 and 2004. Inhibitors of enzymes that are involved in the *de novo* folate cycle are known as anti-folates, for example, thymidylate synthase inhibitors, dihydropteroate synthase inhibitors, and serine hydroxymethyltransferase inhibitors.[47]

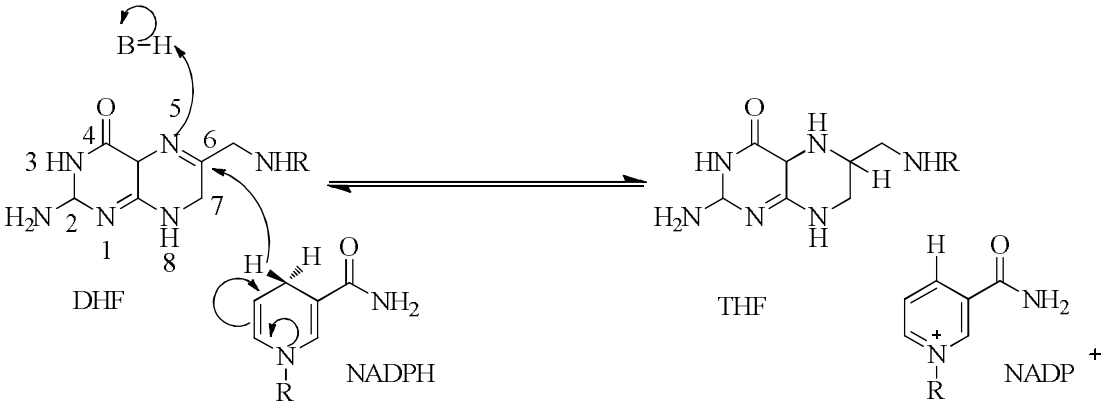
The active site of the mammalian DHFR is larger than the bacterial DHFR. In human DHFR the pteridine ring is attached in the opposite orientation in comparison to other DHFRs. The amino group on the human DHFR forms only one H-bond with amino acid residues in the active site, while bacterial DHFRs form two H-bonds. There are approximately 167 structures of bacterial DHFRs with various ligands bound in the PDB "protein data bank".[47]

Other enzymes involved in *de novo* folate cycle have also been investigated as anti-tuberculosis drugs. For example, dihydropteroate synthase (DHPS) is a protein located in the bacterial cytoplasm; it is the target for sulfonamide and sulfone drugs. Many studies have investigated *Escherichia coli, Staphylococcus aureus,* and *M. tuberculosis* DHPS enzymes as drug targets.[47]

Another enzyme that has been investigated is thymidylate synthase, which is responsible for utilising N^5, N^{10}-methylene THF. Inhibition of thymidylate synthase causes thymineless death, and this is a therapy for cancer. Many studies have examined the structure and mechanism of thymidylate synthase. There are 114 structures of thymidylate synthase in PDB "Protein data bank". However, there are no selective inhibitors for thymidylate synthase. One of the most interesting findings is that some parasites have a bifunctional DHFR-TS enzyme, including *Plasmodium* species, *Toxoplasma gondii,* and *Leishmania.* This finding could be useful in the design of new specific inhibitors for parasites in the future.[47]

Another enzyme that has been studied is serine hydroxymethyl transferase (SHMT), which is responsible for the conversion of serine to glycine using a tetrahydrofolate cosubstrate. Inhibitors of human SHMT have been used to treat cancer, but they have been shown not to be selective for the enzyme. Some DHFR inhibitors, such as triazine anti-folates, have been found to also inhibit SHMT. Some bacteria lack the presence of DHFR enzyme, such as *Helicobacter pylori* and *Treponema palladium,* which also lack the TS gene.[47]

There have been many studies that have focused on the structure and mechanism of DHFR. Czekster *et al.*[48] studied the kinetic and chemical mechanism of *M. tuberculosis.* The DHFR mechanism involves the protonation step at N^5 with the water molecule and transfer of a hydride anion at C^6. This study investigated the order of the two steps. There are two possible models that could occur. One model is the protonation at N^5 first followed by hydride transfer. The other model is that O^4 is protonated first, which leads to a hydride transfer to C^6, followed by N^5 ring protonation. The kinetic isotope effects and the effect of changing pH on rates of both steps suggest a mechanism in which hydride transfer follows the protonation step[48] (Scheme 5).



Scheme 5: Reaction catalyzed by DHFR.

1.2.14.6 Development of new DHFR inhibitors

In the development of DHFR inhibitors, there are three types; The first type comprises the current anti-folate drugs. The second type are the newer DHFR inhibitors that are in the market, for instance trimetrexate, which is active against *Plasmodium carinii* and brodimoprim, which is a TMP analogue, but has a longer half-life. The third class of anti-folates is still under investigation; iclaprim, it has a higher activity against gram-positive bacteria, piritrexim, is active against *M. avium* and it is used to treat methotrexate-resistant cells and epiroprim. It is used against *M. ulcerans* in combination with dapson. Epiroprim has a synergistic effect against *M. leprae.* All of these three types of DHFR inhibitors are 2,4-diaminopyrimidine derivatives (Figure 17).[47]

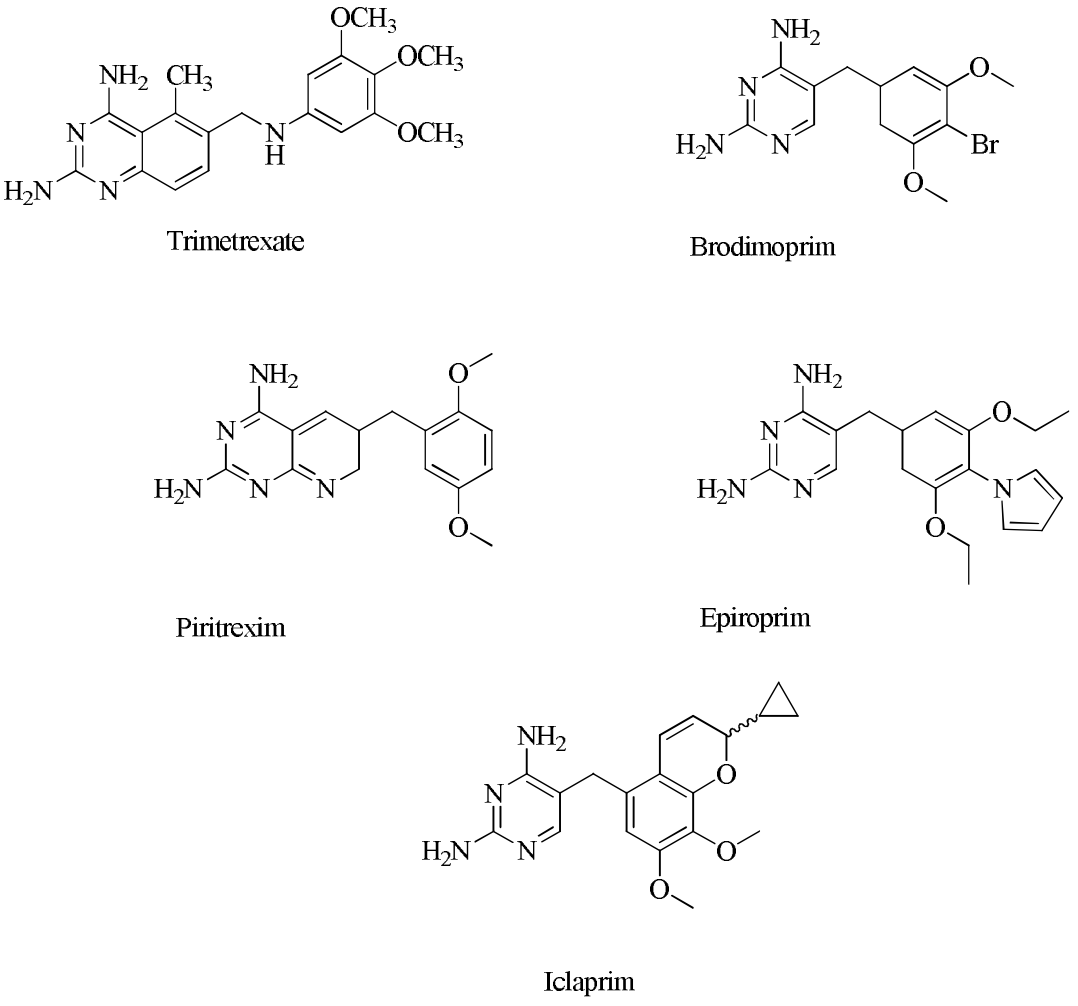


Figure 17: Newly developed 2,4-diaminopyrimidine derivatives.

Diseases currently treated using anti-folates include malaria, pneumonia caused by *Plasmodium carinii*, fungal diseases caused by *Candida albicans*, and protozoal diseases caused by *Toxoplasma gondii*. Because methotrexate is hydrophilic, it has limited access to cells. Lipophilic anti-folates, such as metoprine, piritrexim, and trimetrexate, are also useful for treating resistant cells.[44] Bag *et al.*[49] investigated novel DHFR inhibitors of opportunistic microorganisms, including *Plasmodium carinii*, *Toxoplasma gondii*, and *Mycobacterium avium*, by synthesizing novel compounds that mimic the pharmacophore of anti-folates (such as the 2, 4-diaminopyrimidines), followed by biological evaluation. Computational studies were applied to investigate the binding modes of these drugs and their interactions with the enzyme.[49]

1.2.14.7 Classification of antifolates based on their structures

There are two structural classes of anti-folates: first, classical anti-folates, which are analogues of folic acid and contain an acidic moiety; Second, non-classical anti-folates, which are lipophilic folic acid analogues that lack the glutamic acid moiety. An example of a non-classical anti-folate is epiroprim, which was tested against *M. tuberculosis* and had no activity. Triazine DHFR WR99210 (Figure 18), however, showed good activity against *M. tuberculosis* without selectivity.[47] Triazines, discovered as anti-folates in 1952,[44] are more potent inhibitors of mammalian enzymes than of bacterial enzymes.[50] Another example of a non-classical anti-folate is 2,4-diaminopyrimidine-5-deazapteridines, which showed good activity against *M. avium*.[47]

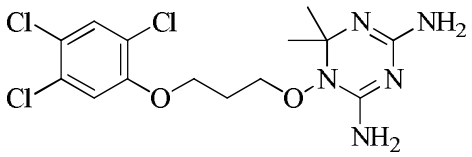


Figure 18: The structure of triazine DHFR WR99210.

Structure activity relationships of anti-folates reveal that the pharmacophore of 2,4-diaminopyrimidine or triazines structures with lipophilic group at position 5 are important for flexibility.[50] Pyrimidines like 5-benzyl-2,4-diaminopyrimidine are the largest and most potent of the 2,4-diaminopyrimidine derivatives. Quinazolines are another nonclassical anti-folate, they are more lipophilic than methotrexate. Quinazolines and methotrexate have an antileukemic effect.[44]

1.2.14.8 The dihydrofolate-binding site

Many studies have shown that lipophilic 2,4-diaminopyrimidines are potent DHFR inhibitors. They are being used as lead compounds for development of novel anti-folate inhibitors.[51] These 2,4-diaminopyrimidines, however, unfortunately lack selectivity against many pathogens.[43]

To describe simply the binding site of the DHFR inhibitor to the *M. tuberculosis* enzyme, the most common feature is the formation of many hydrogen bonds with different protein residues. Compared to the human enzyme, there are many differences between them, which has led to many studies looking to improve the binding affinities of compounds in the mycobacterial DHFR binding site. There is, for example, a glycerol molecule binding close to N^8 of methotrexate and the aminopyrimidine ring of trimethoprim through hydrogen bonds to Asp27, Gln28 and Leu24 of *M. tuberculosis* DHFR, and the glycerol carbon atoms are in contact with Leu20. Studying the glycerol molecule helps provide a template for developing a functional group that can form three hydrogen bonds with the hydroxyl groups of the glycerol molecule.[38]

1.2.14.9 The differences between mycobacteria and human DHFR

The main differences between the mycobacterial and human DHFR are as follows:

- Human DHFR contains 187 amino-acids while mycobacterial DHFR contains 159.
- The human enzyme is larger than the mycobacterial enzyme.
- A glycerol molecule is bound close to the DHF-binding site of *M. tuberculosis*, as shown by the crystal structure. Although it is not clear whether this glycerol is present in the enzyme in the cell or is an artefact of crystallisation, the ease with which it binds suggests that it is an accessible binding motif. This glycerol forms hydrogen bonds with Asp^{27}, Gln^{28} and Leu^{24}.[38] Glycerol is absent from the structure of the human enzyme; the human enzyme has hydrophobic residues (Leu^{22}, Pro^{26} and Phe^{31}) occupying this space instead. The presence of this glycerol-binding motif allows the design of drugs which bind selectively to the mycobacterial enzyme, while not binding to the human enzyme.[45]

The structural comparison of the mycobacterial with the human DHFR is beneficial to develop a novel selective inhibitors of *Mycobacterium tuberculosis* DHFR.

Because of tuberculosis treatment side effects and poor compliance from tuberculosis patients, studies have focused on the discovery of novel drugs that act as DHFR inhibitors. These studies explore their selectivity and potency in the DHFR binding site.

1.2.14.10 DHFR 3-dimentional structure studies

The protein data bank now has over ten lead compounds for potential treatment of *M. tuberculosis*. Studying the structures of these compounds is important. One method that can be used to study and examine the binding interactions of these compounds, is structural interaction fingerprint (SIF). Kumar *et al*.[52] used SIF for mycobacterial DHFR virtual screening. The three known classes of mycobacterial DHFR inhibitors used in the screening showed the same binding signature at the hydrophobic pocket and the heteroatom ring, bearing the N atom bound to active site residues via H-bonding. These inhibitors were 2,4-diaminopyrimidines, deazaptridine derivatives, and triazines. Also, the phenyl side-chains of these inhibitors bind to the protein by hydrophobic contacts with amino acids. Therefore, the newly discovered compounds should have the same pharmacophore and binding interactions. This method of screening is the quickest way to identify compounds that interact with the enzyme.[52]

Gargaro *et al.*[53] studied the structure of *Lactobacillus casei* DHFR using NMR techniques in complex with methotrexate. Previous studies have combined NMR with X-ray crystallography to determine the binding site interactions, and this study was the first to investigate the binding site interactions using NMR techniques only. The structure of *Lactobacillus casei* DHFR with methotrexate obtained by NMR spectra was compared with the corresponding X-ray structure. The study concluded that NADPH binds more tightly to the DHFR-methotrexate complex than to DHFR alone. This kind of investigation will open the gate for further studies to understand binding specificity via different methods other than X-ray crystallography.[53] Methylbenzoprim (Figure 19) is a potent anti-folate which binds more tightly to human enzymes than to bacterial enzymes. Studies have investigated the conformations of methylbenzoprim at the active site of human enzyme using molecular modelling and X-ray crystallography to increase the selectivity of methylbenzoprim analogues for bacterial enzymes.[54]

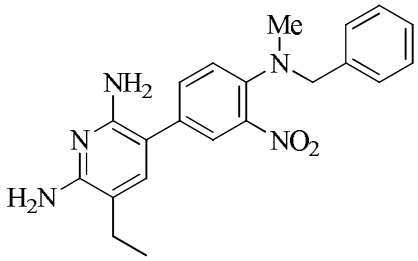


Figure 19: The structure of methylbenzoprim.

2. Aim and objectives

This project focuses on the development of a novel selective inhibitor of *M. tuberculosis* DHFR, based on that reported in a previous study.[45] The designed molecules will incorporate those structural features known to be important for tight binding to DHFR and for selectivity towards the *M. tuberculosis* enzyme. Thus the target compounds will all contain a 2,4-diaminopyrimidine moiety, a common feature of potent DHFR inhibitors (Figure 19). A glycerol moiety will also be included in the inhibitor, as this will promote binding to the *M. tuberculosis* enzyme and disfavour binding to human DHFR, thus allowing selective inhibition of the mycobacterial enzyme. Binding of this glycerol moiety is mediated by a number of hydrogen bonds. The X-ray crystal structure of *M. tuberculosis* DHFR complexed with methotrexate and glycerol will be used to estimate the distance of the spacer unit for the inhibitor glycerol moiety. Examination of DHFR inhibitors such as trimethoprim and pyrimethamine, which are selective for bacterial enzymes, shows the presence of aromatic substitutents which are important for binding. The chance of interactions between this aromatic ring and hydrophobic residues within the active site is very high.[45]

2.1 Aim of the project

The aim of this project is to develop a lead compound that will bind tightly and selectively to *M. tuberculosis* DHFR. The project will focus on exploring the DHFR binding site using a linker at position 6 containing either a O, N or S atom. The synthesis of 2,4-diaminopyrimidines derivatives leading to 5-phenyl-2,4-diaminopyrimidine derivatives with different side chains at position 6 of the pyrimidine ring will be explored.

2.2 Objectives

1. Investigate synthesis of the 2,4-diamino-5-phenyl pyrimidine and trihydroxy alkyl side-chain (‘glycerol’) motifs followed by linking the two motifs together;

2. Investigate synthesis of 2,4-diamino-5-phenyl pyrimidine with an amino group at position 6 and elaboration of the structure with various side-chains.

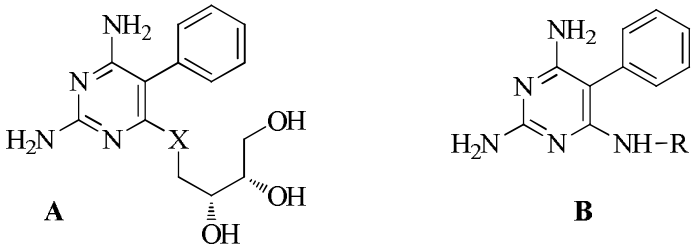


Figure 20: A: The first target compound. B: The second target compounds.

3. Results & Discussion

3.1 El-Hamamsy's research

The aim of this project is to develop a selective DHFR inhibitor of *M. tuberculosis*. The idea has come from previous research by El-Hamamsy,[45] who successfully designed a lead compound (Figure 19) with the main characteristics of an inhibitor of DHFR in *M. tuberculosis.* The design of this compound (Figure 21) was a result of studying the binding site of DHFR in *M. tuberculosis.*[45]

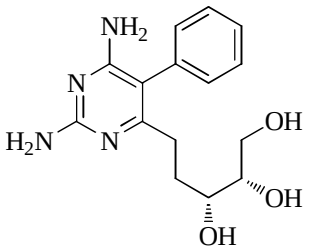


Figure 21: El-Hamamsy lead compound.

Studying the binding site of DHFR in *M. tuberculosis* showed significant features, including: the glycerol pocket in the binding site of the DHFR enzyme and how this glycerol is binding to the amino acids in the same site of DHFR *via* three hydroxyl groups. The lead compound that was synthesised by El-Hamamsy (Figure 21) consists of a 2,4-diaminopyrimidine moiety, which is known to be selective and potent for a DHFR inhibitor.[45]

Because of the waxy layer in the cell wall of *M. tuberculosis*, the penetration of the targeted compound will be difficult. To overcome the penetration problem, the introduction of a lipophilic group such as phenyl group at position 5 of the pyrimidine ring was incorporated by El-Hamamsy, the phenyl group will enhance the lipophilicity of the compound and facilitate the diffusion through the waxy layer of *M. tuberculosis.* Another benefit from the introduction of the phenyl group at position 5 of the pyrimidine ring is enhancing the binding of the compound to the hydrophobic pocket of DHFR.[45]

To enhance the binding affinity to the glycerol pocket in the *M. tuberculosis* DHFR binding site, the trihydroxypentyl side chain was synthesised by El-Hamamsy, and linked to the pyrimidine ring at position 6 (Figure 21). The three hydroxyl groups bind to the amino-acids in the binding site the same way as the glycerol does by forming hydrogen bonds**.**[45]

El-Hamamsy had done modelling studies for the compound (Figure 21) and the crystal structure of DHFR in *M. tuberculosis* has shown that the perfect linker between the 2,4-diamino-5-phenylpyrimidine and the 3,4,5-trihydroxypentyl that mimics the glycerol unit in DHFR binding site of *M. tuberculosis,* is a two carbon chain ($-CH_2CH_2$) linked to position 6 of the pyrimidine ring.[45]

3.1.2 El-Hamamsy's target compounds

El-Hamamsy synthesised a series of 2,4-diaminopyrimidines **(1)–(6)** (Figure 22-26) with a two carbon linker to the glycerol-mimicking triol (3,4,5-trihydroxypentyl) at position 6 of the pyrimidine ring; the 2,4-diaminopyrimidine unit in these compounds carried an aryl substituent at position 5. These features were based on structure-based design.

El-Hamamsy designed a lead compound after modelling the location of the DHFR binding site of *M. tuberculosis* (Figure 22), finding the stereochemistry of the designed compounds **(1)–(6)** is an important factor for the 3,4,5-trihydroxy unit to mimic the glycerol molecule in the binding site, *R* configuration at C-3 and an *S* configuration at C-4 of the secondary alcohol of the 3,4,5-trihydroxypentyl unit were expected to give the same binding properties of the original glycerol molecule in the binding site of DHFR in *M. tuberculosis* **(1).**[45]

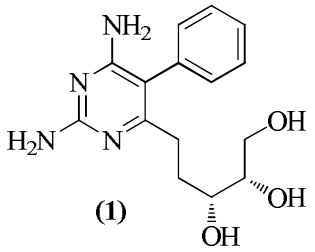


Figure 22: (3*R*,4*S*)-3,4,5- trihydroxypentyl compound.

El-Hamamsy also designed other derivatives by changing the configuration at C-3 to an *S* configuration with the same length of the linker (-CH_2CH_2), to test if the design approach was valid (Figure 23)**.**[45]

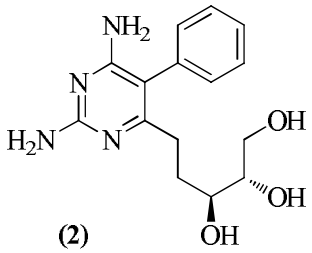


Figure 23: 6-(3*S*,4*S*)- 3, 4, 5- trihydoxypentyl compound.

El-Hamamsy also investigated the importance of the 3,4,5-trihydroxy groups in the lead compound (Figure 19), replacing it by introducing a 6-(5-hydroxypentyl) on the pyrimidine ring instead of 3,4,5-trihydroxypentyl **(3)** (Figure 24)**,** which means there is no hydrogen bonding between the glycerol mimic unit (6-(5-hydroxypentyl) and the amino acids (Trp22, Asp27 and Gln28) that were shown in the glycerol binding site of the DHFR.[45]

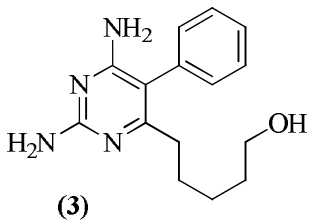
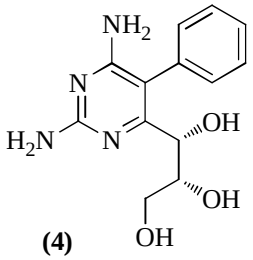


Figure 24: 6-(5-hydroxypentyl) compound.

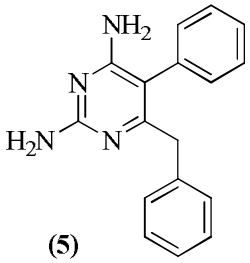
Another modification designed by El-Hamamsy, changed the orientation of the 3,4,5-trihydroxypentyl group directed to the pyrimidine ring with a shorter carbon-chain length **(4)** (Figure 25)**.**[45]



(4)

Figure 25: 6-(1*S*,2*R*)-1,2,3-trihydroxypropyl compound.

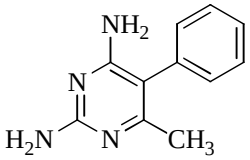
El-Hamamsy also designed other 5-phenyl-2,4-diaminopyrimidine compounds, one by replacing the 3,4,5-trihydroxypentyl group by a benzyl group **(5)** (Figure 26)**,** which might have an unfavourable interaction in the hydrogen bonding environment of the glycerol-binding pocket in the DHFR binding site of *M. tuberculosis.*[45]



(5)

Figure 26: 6-benzyl-5-phenylpyrimidine-2,4-diamine.

The other compounds that were designed removed any group that would occupy the glycerol pocket **(6)** (Figure 27)**.**[45]

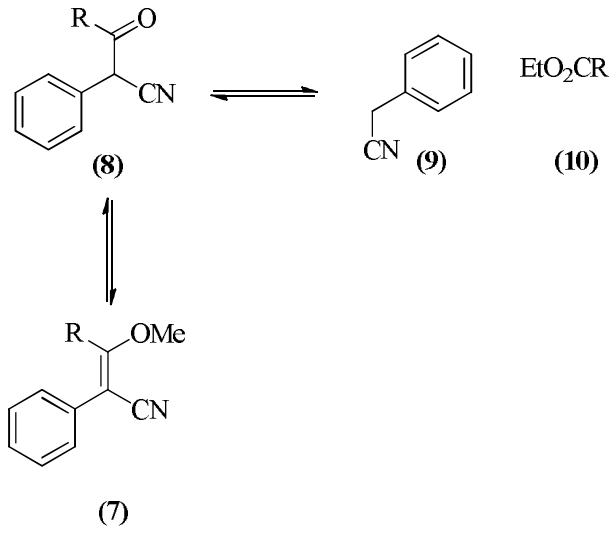


(6)

Figure 27: 6-alkyl compound at position 6.

3.1.3 El-Hamamsy's synthetic strategy for 5-phenyl-2,4-diaminopyrimidne compounds

El-Hamamsy's synthetic strategy for the 2,4-diaminopyrimidine unit in compounds **(1-6)** was the condensation of a substituted enol ether, appropriate for synthesis of each of these compounds **(1-6)**, with guanidine. The enol ethers **(7)** were prepared by methylating the α-acylphenylacetonitrile **(8)** that resulted from the reaction of an Ar-substituted phenylacetonitrile **(9)** with the appropriate ester **(10)** for each compound's synthesis (Scheme 6).[45]



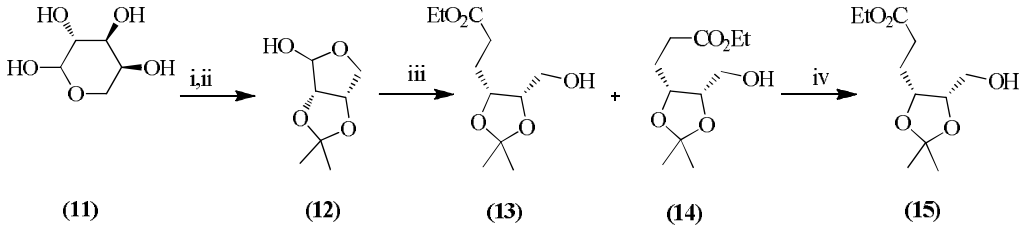
Scheme 6: El-Hamamsy's retrosynthetic strategy of 2,4-diaminopyrimidine compounds.
R= 3*R*,4*S*; 3*S*,4*S*; 1S,2*R*-1,2,3,trihydroxypentyl, $Ph(CH_2)_2$, Me.

3.1.4 El-Hamamsy's synthetic strategy for 3, 4, 5-trihydroxypentyl compounds

El-Hamamsy's plan was synthesis of two building blocks, the 2,4-diaminopyrimidine and the 3,4,5-trihydroxypentyl group, followed by linking the two units. Synthesis of the 3,4,5-trihydroxypentyl unit from the ester **(15),** started with acetonide protection of the *cis* 3-OH and 4-OH of arabinose **(11),** followed by oxidation cleavage of the (C-1, C-2) bond with periodate to give the protected *L*-erythrose **(12)** (Scheme 7).[45]

To achieve the two carbon chain extension at position 6 for compounds **(1-6),** El-Hamamsy managed to apply Wittig reaction of the aldehyde with ethyltriphenylphosphoranylidineacetate to afford the stereoisomeric α, β -unsaturated

esters **(13), (14).** These isomers were readily separated by chromatography but it was not necessary as the hydrogenation of the mixture gave the saturated ester **(15)** quantitatively (Scheme 7).[45]

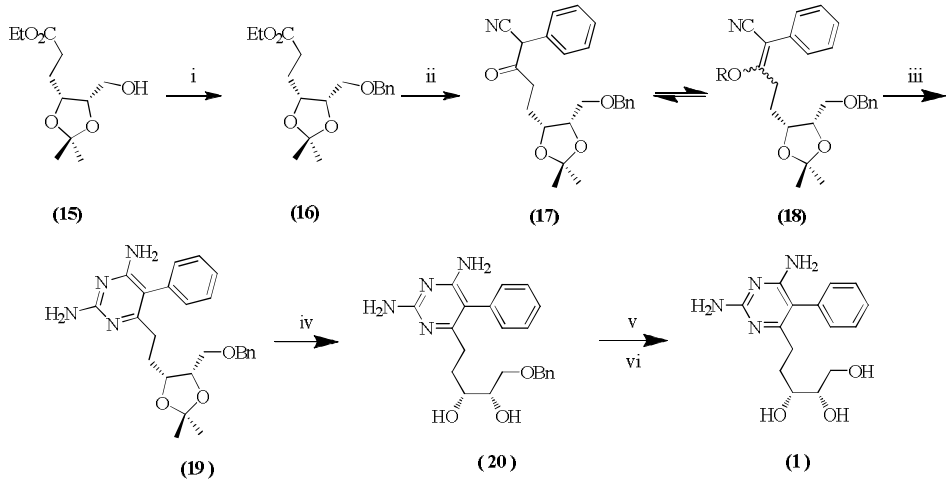


(11) (12) (13) (14) (15)

Scheme 7: El.Hamamsy's synthetic routes to 3,4,5-trihydroxypentyl unit. Reagents: i:$Me_2C(OMe)_2$, TsOH, DMF; ii:$NaIO_4$, H_2O, hexane; iii: $EtO_2CCH{=}PPh_3$, CH_2Cl_2; iv: H_2, Pd/C, EtOH.

3.1.5 Synthetic route to target compound (1)

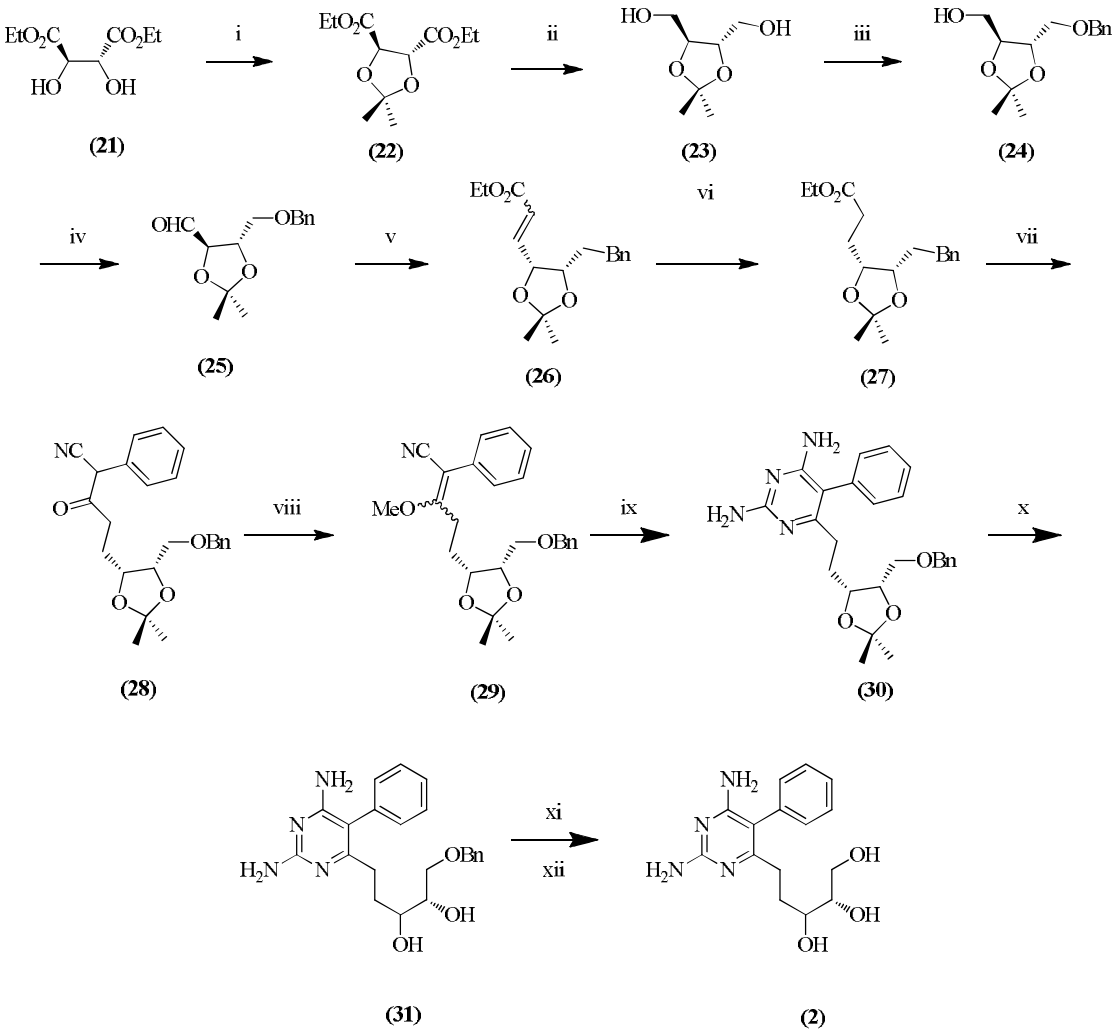
In order to synthesize **(1)**, El-Hamamsy investigated many protecting groups for the primary alcohol **(15),** finally benzylation was chosen. Benzylating the hydroxyl group to give the fully protected ester **(16)**, was affected by deprotonation with lithium bis(trimethylsilyl)amide and alkylation with benzyl bromide. The α-acylphenylacetonitrile **(17)** was generated from reaction of the phenylacetonitrile and the protected ester **(16)** with lithium bis(trimethylsilyl)amide. This was followed by methylation with diazomethane to give the enol ether **(18)** (as a mixture of geometrical isomers). Condensation of this isomeric mixture with guanidine gave the required 2,4-diaminopyrimidine **(19)**. After joining the required units, El-Hamamsy had investigated many ways to remove the protecting groups, such as the removal of the acetonide with aqueous trifluoroacetic acid to give compound **(20)**. Also El-Hamamsy investigated the removal of the benzyl group by catalytic hydrogenation with chloroform, unfortunately, it failed to remove the benzyl group. El-Hamamsy managed to debenzylate **(1)** after trying different methods. The reduction of the O-benzyl with sodium in ammonia succeeded but because it is not applicable for the halogen-substituted compounds, which was El-Hamamsy's intention, El-Hamamsy applied the reaction with the Lewis acid anhydrous iron(III) chloride in dichloromethane (Scheme 8).[45]



Scheme 8: Synthetic routes to compound (1).
Reagents: i: $LiN(SiMe_3)_2$, BnBr, THF, DMF; ii:CH_2N_2, Et_2O; iii: guanidine.HCl, NaOMe, $MeO(CH_2)_2OH$; iv: aq CF_3CO_2H; v:Na, liquid NH_3 or $FeCl_3$, CH_2Cl_2.

3.1.6 Synthetic route to target compound (2)

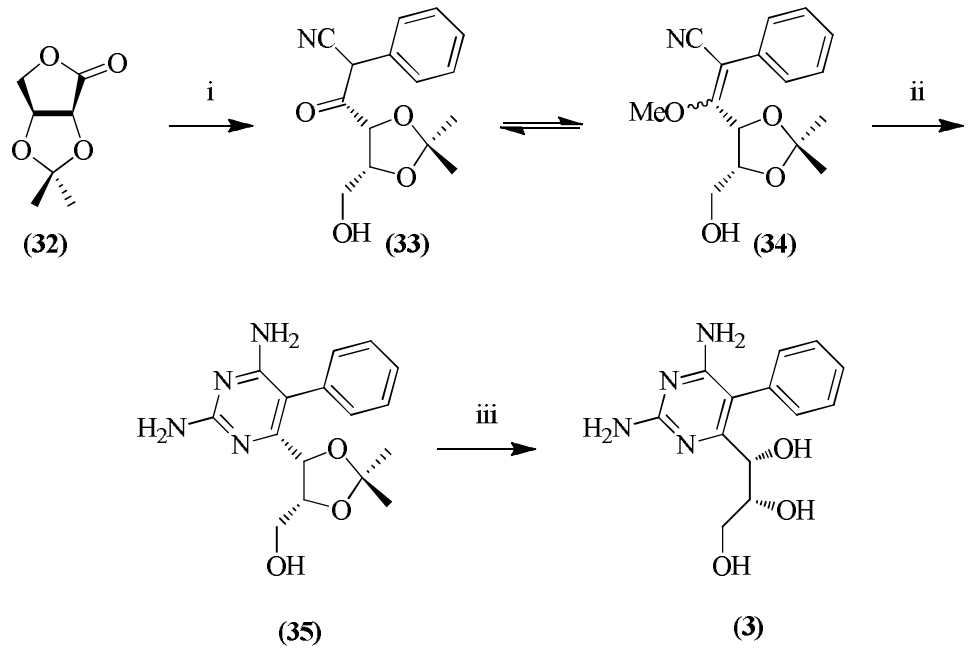
El-Hamamsy obtained **(2)** by following the same strategy as compound **(1)** but by starting with diethyl *trans-R,R*-tartrate **(21)** followed by acetonide protection **(22)**, then reduction with lithium aluminium hydride to obtain the C-2 symmetric diol **(23)**. The mono-protection of **(23)** was developed with sodium hydride in dimethylformamide (DMF) followed by benzyl chloride alkylation protection to give **(24)**. This was then oxidised by pyridinium chlorochromate to give the aldehyde **(25)**, then the Wittig reaction was applied by condensing the aldehyde **(25)** with ethyl triphenylphosphoranylidineacetate to give again the chain-extended α,-β-unsaturated ester **(26)**. Then a hydrogenation in the presence of palladium reduced the alkene **(26)** to form **(27)**. This was treated the same way as (Scheme **1**) to give **(29)**, acid hydrolysis removed the acetonide to give **(31)** and iron (III) chloride carried out the debenzylation to get the other lead compound **(2)** and the deprotection was applied (Scheme 9).[45]



Scheme 9: Synthetic routes of compound (2).
Reagents: : i: 2,2-dimethoxypropane, TsOH: ii, $LiAlH_4$; iii: BnCl, NaH, DMF. iv: PCC, NaOAc, CH_2Cl_2, v: $EtO_2C{=}PPh_3$, $PhCO_2$, PhMe. vi: H_2, Pd/C, EtOH. vii: $LiN(SiMe_3)_2$, BnBr, THF, DMF; viii:CH_2N_2, Et_2O; ix: guanidine.HCl, NaOMe, $MeO(CH_2)_2OH$; x:aq CF_3CO_2H; xi: H_2, Pd/C; xii: $FeCl_3$, CH_2Cl_2.

3.1.7 Synthetic route to target compound (3)

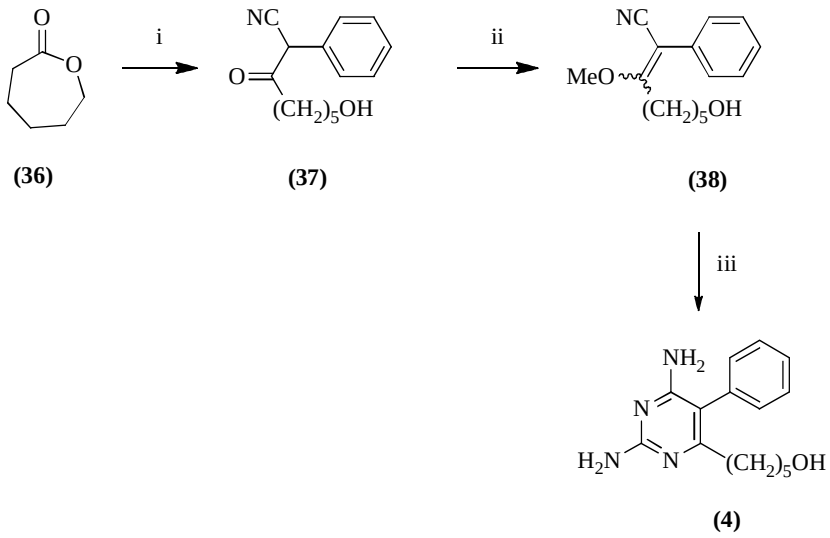
Formation of **(3),** in which the carbon chain was shortened**,** followed the same condensation with guanidine, methylation with diazomethane then the deprotection strategy, starting from condensation of *L*-erythrolactone **(32)** with phenylacetonitrile to give **(33)**, then methylation to give the enol ether **(34),** which then condensed the same way with guanidine. The acetonide was removed in the same way in aqueous acid to give **(3)** (Scheme 10).[45]



Scheme 10: Synthetic route of compound (3).Reagents: ii: $LiN(SiMe_3)_2$, $ArCH_2N_2$, Et_2O; ii: CH_2N_2, Et_2O; ii: guanidine.HCl, NaOMe, $MeO(CH_2)_2OH$; iii: aq CF_3CO_2H.

3.1.8 Synthetic route to target compound (4)

The lactone strategy was also applied to form **(4)** a 6-(5-hydroxypentyl)pyrimidine-2,4-diamine. Condensation of a lactone **(36)** with lithium bis(trimethylsilyl)amide and phenylacetonitrile gave **(37)** in very low yield; then methylation of **(38)** with diazomethane and condensation with guanidine gave **(4)** (Scheme 11) and no deprotection steps were involved.[45]

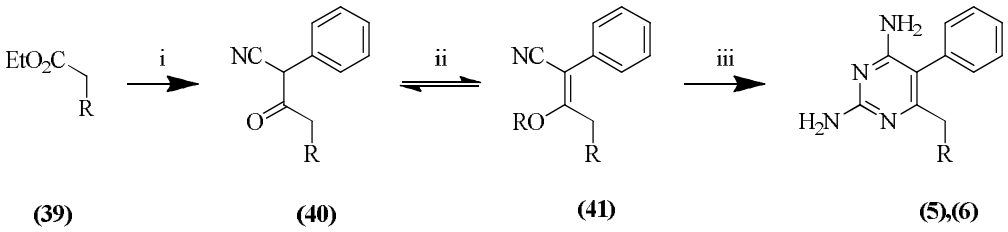


(36) **(37)** **(38)** **(4)**

Scheme 11: Synthetic route of compound (4).
Reagents: ii: $LiN(SiMe_3)_2$, $PhCH_2N_2$, Et_2O; ii: CH_2N_2, Et_2O; iii: guanidine.HCl, NaOMe, $MeO(CH_2)_2OH$.

3.1.9 Synthetic route to target compounds (5) and (6)

The synthesis of the 2,4-diaminopyrimidines **(5)** and **(6)** with no alcohol at position 6 was approached by the same strategy as before (Scheme 6) with condensation and then methylation.[45]



(39) **(40)** **(41)** **(5),(6)**

Scheme 12: Synthetic route to compound **(5)** and **(6)**.
Reagents: i: $LiN(SiMe_3)_2$, $ArCH_2N_2$, Et_2O; ii: CH_2N_2, Et_2O; iii: guanidine.HCl, NaOMe, $MeO(CH_2)_2OH$. R= Bn, H, Me.

Acylation of phenylacetonitrile with ethyl 2-phenylpropanoate **(39)** gave **(40)**, which was then methylated with diazomethane to give **(41)**. The usual way of the condensation with guanidine formed **(5)**, while **(6)** was formed by acylating phenylacetonitrile with ethyl acetate (Scheme 12).[45]

3.1.10 El-Hamamsy's biological studies and conclusion

After designing and synthesising these series of compounds **(1-6)**, El-Hamamsy had carried out indirect biological studies to test these compounds, as testing these compounds directly against *M. tuberculosis* directly requires biosafety level 3 procedures. El-Hamamsy evaluated the inhibitory effect of these compounds against the growth of yeasts containing human and *M. tuberculosis* DHFRs. Only compound **(1)** has selectivity and activity against *M. tuberculosis* DHFR, while the others varied from modest to no activity. El-Hamamsy found that the configuration is critical for selectivity. The presence of the secondary alcohol at position 6 of the pyrimidine ring is necessary for binding to the glycerol-binding pocket. The length of the linker is also critical as compound **(3)** with shorter extension decreased the activity against *M. tuberculosis* DHFR. Compound **(1)** was also shown to be the most selective by modelling; it binds in the same mode as methotrexate in the binding site.[45]

El-Hamamsy had achieved the best conformation for the compound **(1)**; the phenyl group had to be twisted by rotating it anticlockwise out of the plane. It fit well into the site as the rotation clockwise from the plane showed that the edge of the phenyl ring was pressed against the top of the enzyme very tightly.[45]

3.2 Discussion of the current project

The lead compound **(1)** that had been designed by El-Hamamsy is the current focus of this project; aiming to modify it to be more selective and more flexible at the binding site, the new plan is to look for other strategies to make the synthesis easier, such as replacing the ($-CH_2$) of the linker that is adjacent to the pyrimidine ring with an oxygen, nitrogen or sulfur. Oxygen will sterically mimic CH_2 closely, whereas an NH will be sp^2 hybridised and introduce some rigidity, sulfur is slightly larger than CH_2 and will locate the trihydroxy unit further away.

3.2.1 Synthesis of 5-phenyl-2,4-diaminopyrimidine motif

3.2.1.1 Diaminopyrimidines role

As our compound is one of the 2,4-diaminopyrimidine derivatives, there are many studies that have focused on their structures, synthesis and their potency against many diseases that are caused by specific microorganisms or tumours.[55]

Many natural and synthetic biomolecules have interesting properties because of the pyrimidine ring in their structures, which plays an important structural role. These biomolecules are the nucleotide bases components in DNA and RNA of living cells; vitamins; coenzymes; and also some drugs, such as sulfadiazine and fluorouracil.[55]

3.2.1.2 Diaminopyrimidines classifications and structures

Hitchings *et al.*[56] reported that diaminopyrimidines are classified into two types in terms of potency and structure according to studies that focused on folic acid antagonists in the growth of *Lactobacillus casei*: the substituted diaminopyrimidines at position 5 and the unsubstituted diaminopyrimidines. The substituted diaminopyrimidines are one hundred times more potent than the unsubstituted diaminopyrimidines.[56] One of the properties of the diaminopyrimidine structures is that diaminopyrimidines have structural and geometrical similarities to the folinic acid and its metabolites, these similarities include: the disubstituted pyrimidine and the benzene rings. Any modification on the chemical structure of the diaminopyrimidine may change their functions and make them specific against particular species and tissues. This is the main way for finding a treatment target.[56]

Studying the exact chemical structure of the diaminopyrimidines and their affinity to the receptors on the organisms has led to knowing certain conditions and specifications that play a key role on the modifications of the substituents on the pyrimidine ring at position 5 and 6 (Figure 28).[56]

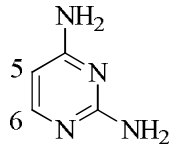


Figure 28: 2,4-diaminopyrimidine.

Another property for aminopyrimidine and its derivatives is that they are able to form stable hydrogen bonds through the amine groups on the pyrimidine ring, this hydrogen bonding is important in molecular recognition, especially in biological systems.[55]

3.2.1.3 Diaminopyrimidine derivatives studies

A 2-aminopyrimidine and its derivatives, have a broad spectrum activity against many microorganisms. The organic synthesis of these compounds has been of interest for years, and many studies have shown different methods for the synthesis of these compounds, and this was apparent in the introduction of the Glivec® (Imatinib) (Figure 29) in 1995 as 2- aminopyrimidine kinase inhibitor.[55]

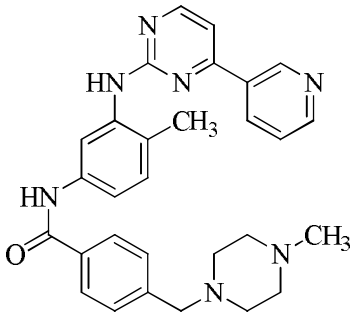
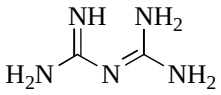


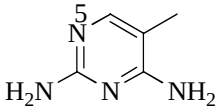
Figure 29: The structure of Glivec®.

The introduction of Glivec® was one example for many studies that found out about the uses of the 2,4- diaminopyrimidines as antitumour.[55]

Hitchings *et al.*[56] introduced a suggestion according to the similarities in the chemical structure between the substituted diaminopyrimidines at position 5 **(47)** and the antimalarial biguanides **(48)** (Figure 30), that the 5-substituted diaminopyrimaidine might have some antimalarial activity.[56]



(48)



(47)

Figure 30: **47**; 5-substituted 2,4-diaminopyrimidine; **48**: Biguanide.

Hitchings *et al.*[56] also investigated how the modified substituents at position 5, 6 in the diaminopyrimidines will show some activity against *Streptococcus faecalis* and *Lactobacillus casiae* as they inhibit the folic acid or folinic acid, and it has been found that 5-phenoxy-2,4-diaminopyrimidine (Figure 31) are more active as folic acid antagonist in *Lactobacillus ceasie*. Also some studies have shown that the diaminopyrimidines have some activity against *Plasmodia* and *Taxoplasma* because they are antimetabolites of the folic acid.[56]

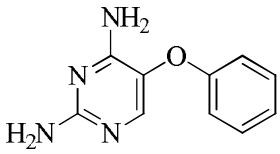
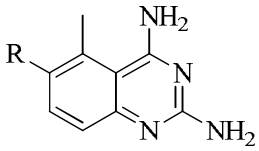


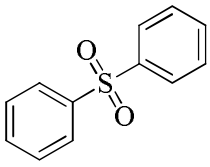
Figure 31: 5-phenoxy-2,4-diaminopyrimidine.

Other diaminopyrimidine derivatives that have been found with some antibacterial effects are diaminopyrimidine substituted at position 6; they have shown some activity against the DHFR in *M. leprae* and *M. sp.* 607 but there is no mycobacterial cell growth inhibition and this was an inspiration to study the fused bicyclic pyrimidine system against the *Mycobacterium* species such as *M. sp. 607*, for example, fused bicyclic pyrimidines and 2,4-diamino-5-methyl-6-alkylquinazolines **(49)**. Compound **(49)** has shown moderate to strong growth inhibition of *M. sp.* 607. By investigating different 2,4-diamino-5-methyl-6-alkylquinazolines, those investigators found out that the activity increased with alkyl groups such as propyl quinazoline and 6- propyl analogues, and it also **(49)** has a synergistic effect against *M. sp. 607* with dapsone (bis(4-aminophenyl)sulfone) **(50)** (Figure 32).[58]



(49)

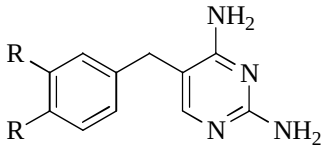
R= $(CH_2)_3CO_2H$.



(50)

Figure 32: **49**: 2.4-diamino-5-methyl-6-alkylquinazolines; **50**: diaminopyrimidine diphenyl sulfone.

For antimicrobial activities, some derivatives of 5-benzyl-2,4-diaminopyrimidine have been investigated by Roth *et al*.[59] Two interesting findings came from that study. First, the unsubstituted-6-diaminopyrimidines showed the highest activity. Second, the substituted benzyl at position 5 of the diaminopyrimidines with one or more alkoxy groups at *meta* and *para* positions **(51)** (Figure 33) showed a strong activity against gram positive organisms and some activity against several gram negative organisms.[59]

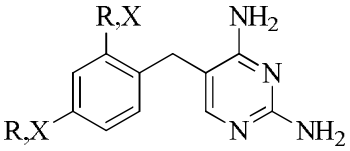


(51)

R= alkoxy

Figure 33: Substituted benzyl at position 5 of the diaminopyrimidines with one or more alkoxy groups at meta and para positions.

The aim of some studies was looking for more substituted 5-benzyl-2,4-diarninopyrimidines to broaden the activity spectrum against gram negative bacteria. Some of the results of these studies was the synthesis of *p*- and *m*-methoxy, and halo groups on 5 benzyldiaminopyrimidines **(52)** (Figure 34), these showed high activity against *Proteus vulgaris*. This early discovery led to more synthesis of different alkoxy and halo groups at the benzene ring to seek for highly effective and less toxic compounds.[59]

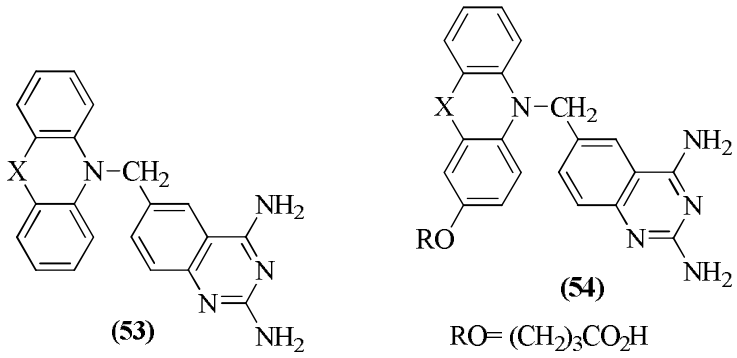


(52)

R= alkoxy
X= Cl, Br, I, Fl

Figure 34: Alkoxy or halo substituted 5-benzyl-2,4-diaminopyrimidinones.

In 1999 there was the discovery of the novel quinzalonediamines as DHFR inhibitor. Their structures are characterised by a 2,4-diaminopyrimidine ring with a lipophilic tricyclic group at position 6. They showed high activity against some bacteria, for example, **(53)** is highly effective against *M. avium* DHFR and **(54)** is active against *Mycobacterium avium, Toxoplasma gondii* and *Pneumocystis carnii* (Figure 35).[60]



(53)

(54)

RO= $(CH_2)_3CO_2H$

Figure 35: Tricyclic substituted 2,4-diaminopyrimidines DHFR inhibitors (quinazolinediamines).

Robson *et al.*[61] studied 12 novel 2,4-diamino-5-aryl-6-ethyl pyrimidines (Figure 36) and assessed these compounds to be non-classical inhibitors of *Pneumocystis carinii* and *Toxoplasma gondii* DHFRs.[61]

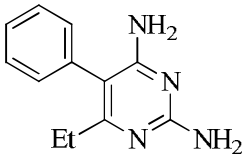
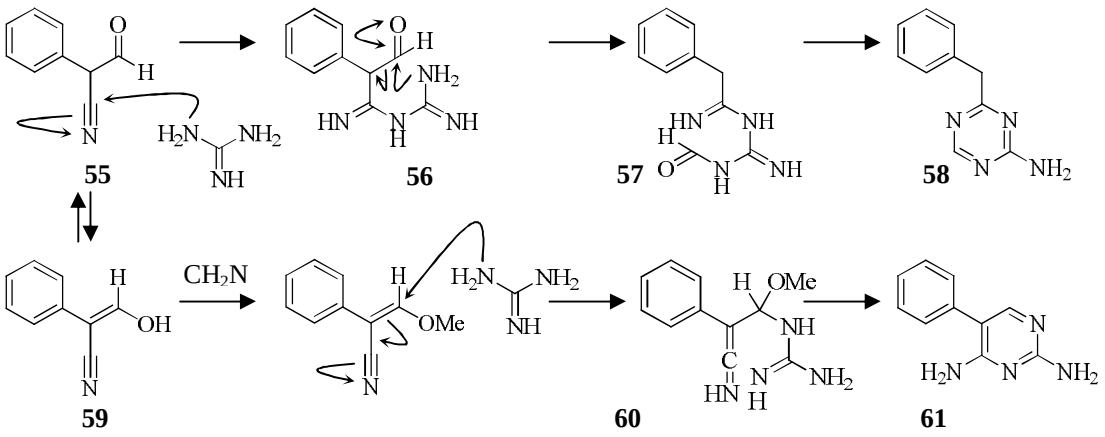


Figure 36: 2,4-diamino-5-aryl- 6-ethyl pyrimidines.

Some studies in the hormones field have found that the 2,4-diaminopyrimidines derivatives are active against Ghrelin, which is a hormone in the stomach and hypothalamus that causes obesity because it stimulates the hunger centre, which leads to more food intake. The study of the synthesis of different substituents has been carried by Serby *et al*.[62] In addition it has been found that the DHFR inhibition is much higher and stronger than Ghrelin hormone inhibition.[62]

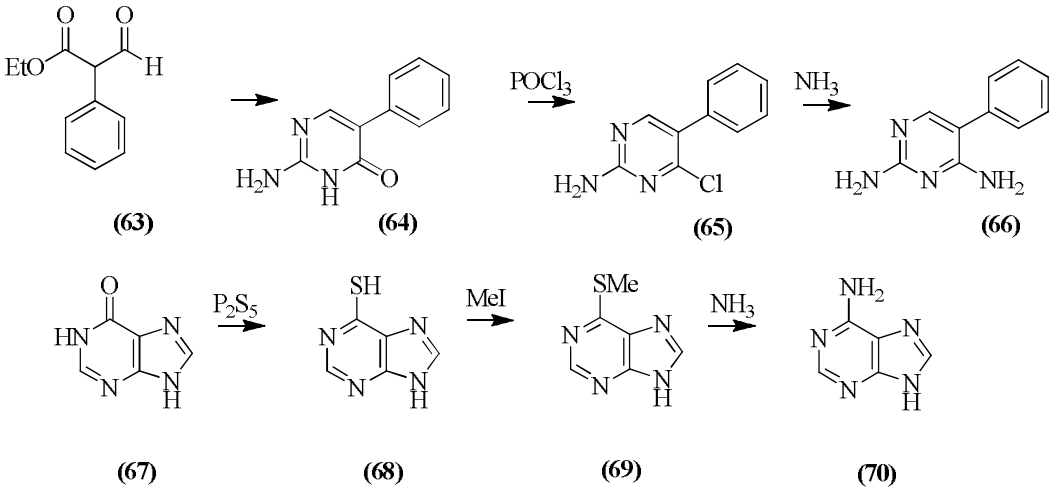
3.2.1.4 Common methods for synthesis of 2,4-diaminopyrimidines

There are many different strategies that have been reported for the synthesis of different 2,4-diaminopyrimidine derivatives. It has been reported that the primary amino group at position 2 can be introduced by using guanidine as the one-carbon fragment. Guanidine supplies the 2-NH_2, the 2-C and the two ring nitrogens. The other primary amino group at position 4 can be introduced by using a nitrile or dinitrile in the three-carbon fragments. Scheme 13 shows one of the studies carried out by Russell *et al*,[63] a condensation of β-aldehydonitriles **(55)** with guanidine. This, however, gave 2-amino-4-benzyl-1,3,5-triazine **(56),** through direct attack of the guanidine on the electrophilic nitrile, followed by a transformylation **(57)** and cyclisation **(58).** This problem was overcome by methylating the β-aldehydonitrile **(59)** with diazomethane to convert it to the enol ether **(60).** Condensation of the enol-ether with guanidine **(61)** then gave 2,4-diaminopyrimidine derivatives **(62)** (Scheme 13). This suggests that the condensation of enol ether with guanidine is easier and takes place by attack of the conjugate electrophile in the unsaturated nitrile.[63]



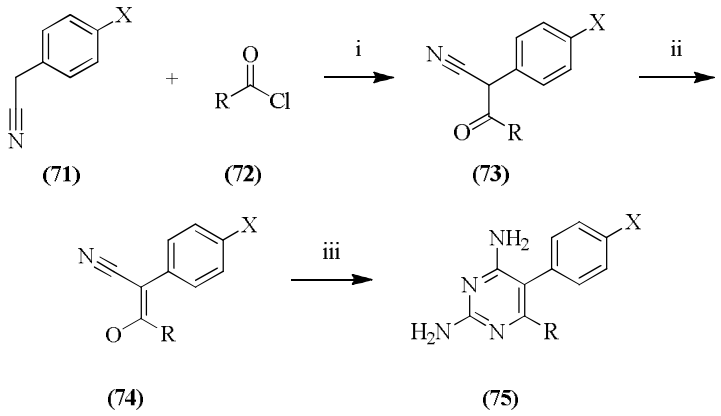
Scheme 13: Condensation of α-formylnitriles and enol ethers with guanidine.

Another strategy for the preparation of 2,4-diamino-5-phenylpyrimidine derivatives by Elion *et al.*[64] is useing an ester instead of a nitrile (Scheme 14). For example, α-formylphenylacetic esters **(63),** if it condensed with guanidine will give 2-amino-4-hydroxypyrimidine **(64).** Chlorination by phosphorus oxychloride **(65)** followed by treatment with ammonia gave the expected 2,4-diaminopyrimidine **(66).** Analogously, purinones **(67)** can be converted to mercaptopurines **(68)** with phosphorus pentalsulfide, these can then be methylated **(69)** and treated with ammonia to give aminopurines **(70)** (Scheme 14).[64] The problem with these two pathways, however, was the very low yields.



Scheme 14: Introducing the amino group via the chloropyrimidine or the mercaptopurine.

Another way to synthesize the 2,4-diaminopyrimidine core was demonstrated by Serby *et al.*[62] They started with acylation of 4-nitrophenylacetonitrile or 4-cyanophenylacetonitrile **(71)** with an acid chloride **(72)** to give an aryl-α-ketonitriles **(73)**; then **(73)** reacted with either trimethylsilyldiazomethane or diazomethane in diethyl ether to give enol ethers **(74)**. By treating **(74)** with guanidine and removing methanol, the 2,4-diaminopyrimidine **(75)** was formed (Scheme 15).[62]



Scheme 15: Condensation with guanidine by Serby *et al.*[62] Regents and conditions:i: Et_3N, DMAP, CH_2Cl_2; ii: $TMSCHN_2$, CH_2Cl_2; iii: guanidine, EtOH, reflux. R= Et, CH_2OCH_2Ph, CH_2CH_2Ph; X= NO_2, CN.

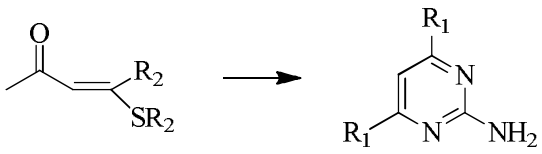
Some modifications have been done for compound **(75)** on the same study by using different kinds of groups by replacing the X group on **(75)** to test them against the growth hormone Ghrelin; and the result was that **(75)** still possesses the highest activity against DHFR.[62]

Koroleva *et al.*[55] have reviewed the literature on the syntheses of 2-aminopyrimidines. They identified two types of route to these compounds, condensation reactions forming the ring from a reagent carrying the 2-NH_2 (or generating the 2-NH_2 during the condensation) and S_NAr reactions in which a leaving group at the 2-position of a pre-formed pyrimidine is substituted by an amine or ammonia. Yields were often low in the latter substitution.[55]

3.2.1.5 Different condensation method with guanidine

There are different methods of pyrimidine ring condensation, depending on the nature of the moieties. Compounds with β-dicarbonyl, such as β-keto esters, and β-keto nitriles, can react with any nucleophile in polar solvent in the presence of a condensing agent such as guanidine. This reaction when it is followed by removal of water or methanol, gives a condensed pyrimidine ring.[57]

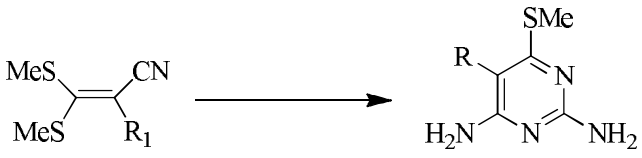
In the 1980s, mono- and di-β-sulfonyl derivatives of α,-β-unsaturated ketones were discovered as starting materials for synthesis of 2-aminopyrimidines. These compounds contain two reactive sites for nucleophilic reaction at C -1 and C -3. The reactions of sulfanyl α,β-enones with guanidine afford the corresponding 2-aminopyrimidines (Scheme 16).[57]



R_1=Me, Ph, 4-MeC_6H_4, 4-$MeOC_6H_4$; R_2=Me, Ph.

Scheme 16: Synthesis of 2-aminopyrimidine.

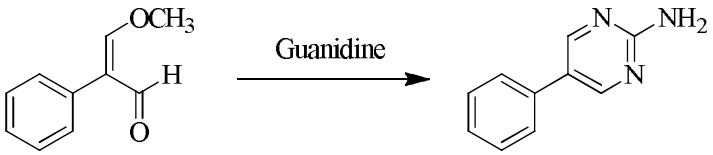
Also, another method for the condensation that had been reported is condensation of α-cyanoketene *S,S*-acetals with guanidine and potassium carbonate to give 2,4-diaminopyrimidines (Scheme 17).[57]



Scheme 17: Condensation of α-cyanoketene S,S-acetals with guanidine and potassium carbonate gives 2,4-diaminopyrimidines.

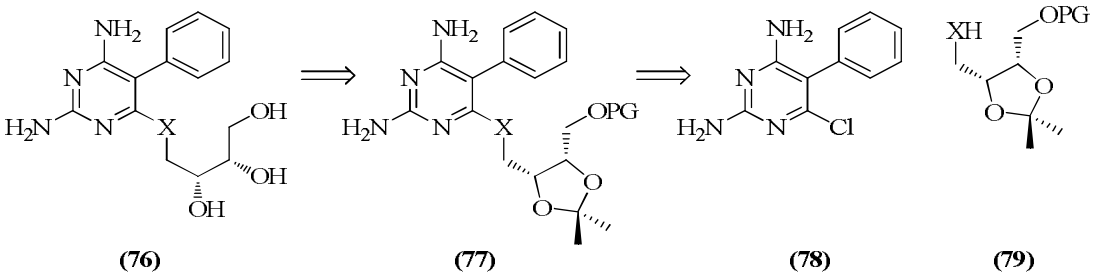
3.2.1.6 Synthetic strategy of 2,4-diaminopyrimidne motif by condensation with guanidine

Our work in in this area began with planning a synthetic strategy of 2,4-diaminopyrimidine motif. Many studies have reported that the condensation of α-formylphenylacetic esters with a group at the *ortho* position leads to unsuccessful reaction.[65] The reason might be the enolisation and the acidity of these β-carbonyl derivatives. Successful condensation of the enol-ethers with guanidine has, however, been reported and proved by Rupe.[66] He introduced the synthesis of 2-amino-5-phenylpyrimidine as shown in Scheme 18. This approach was therefore adopted in the synthesis of the 2,4-diaminopyrimidine derivatives.



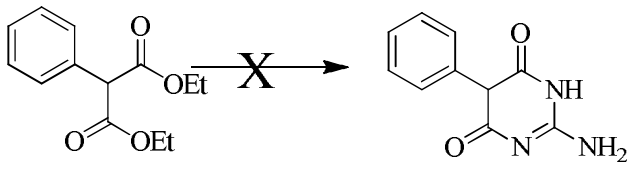
Scheme 18: Condensation of enol ether with guanidine by Rupe.[64]

Scheme 19 shows one of the suggested plans, the retrosynthetic planning for the first target compound **(76)**. This target is formed by deprotection of **(77)**. This key compound is formed from the chloropyrimidinediamine **(78)** and the masked triol unit **(79)**. The other plan was synthesising compound **(78)** but replacing the chloro atom with amino analogues.



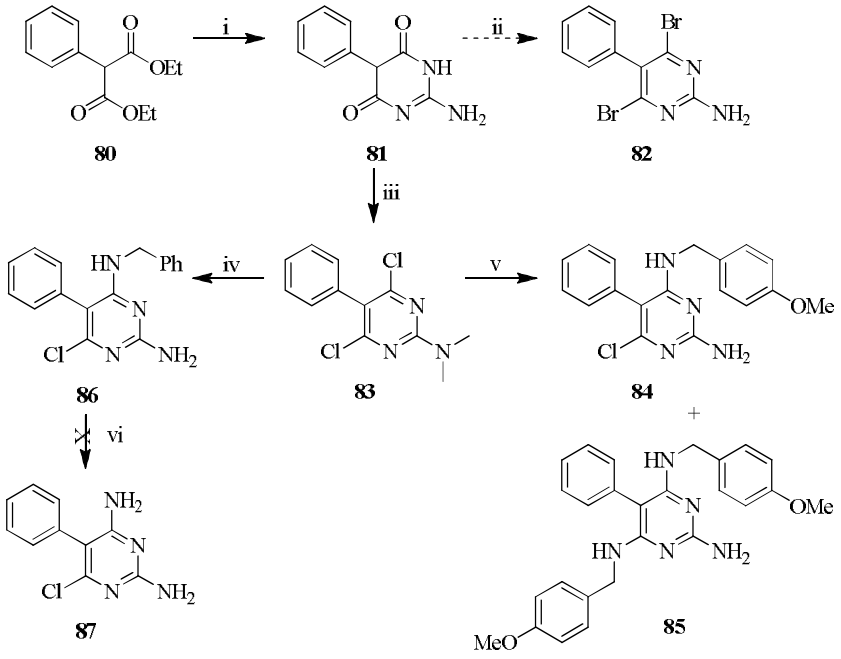
Scheme 19: Retrosynthetic plan for target compound (75), X= O, NH, S.

In the present work, to start with both plans the first steps were the same. The first attempt to synthesise the 2,4-diaminopyrimidine derivative therefore began by treating guanidine hydrochloride with *tert*-butoxide to form the free base and make it a better nucleophile. This was then treated with diethyl phenylmalonate **(80),** which is commercially available (Scheme 20).



Scheme 20: Attempted condensation of the diester **(80)** with guanidine hydrochloride in the presence of potassium *tert*-butoxide. Reagents and conditions: guanidine.HCl, KOBut, EtOH, reflux.

The reaction was monitored with thin layer chromatography (TLC) over five hours without any progress observed. The reaction was repeated using two equivalents of the potassium *tert*-butoxide but the result was the same. Basford[67] carried out the same reaction using sodium methoxide in methanol under reflux. Using his conditions, the reaction was therefore repeated with sodium methoxide under reflux for three hours to give **(81)** in 46% yield (Scheme 21).[67] The reason for this difference in outcome with the different bases is not clear.



Scheme 21: Synthesis of chlorodiaminopyrimidines. Reagents and conditions:
i: Guanidine.HCl, NaH, MeOH, reflux, 3 h, 46%; ii: $POBr_3$, reflux, 3 h; iii, $POCl_3$, reflux, 3 h, 83%;
iv: $PhCH_2NH_2$, AcOH, 130°C, 2 h, 62%; v: 4-$MeOPhCH_2NH_2$, AcOH, 130°C, 2 h, 68%; vi: H_2, Pd/C, various conditions.

3.2.2.2 Halogenation of carbonyl groups 2-Amino-5-phenyltetrahydropyrimidine-4,6-dione / 2-Amino-5-phenylpyrimidine-2,4-diol of (81) by phosphorus oxybromide and phosphorus oxychloride

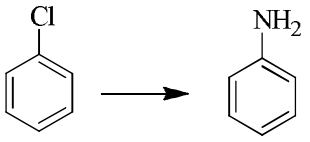
In order to introduce an amino group at position 4 of the pyrimidine ring, the site should be susceptible to nucleophilic attack. Firstly, the synthesis of 2-amino-4,6-dibromo-5-phenylpyrimidine **(82)** was attempted, as bromide is an excellent leaving group for the next step (displacement with an amine). However, treatment of **(81)** with phosphorus oxybromide at reflux gave a crude mixture of **(82)** and products with bromine introduced electrophilically into the benzene ring. Chloride is a weaker leaving group than bromine but may be satisfactory for the next step. Halogenation of the carbonyl groups was carried out by treating compound **(81)** with phosphorus oxychloride under reflux for three hours to give **(83)** in a very good yield (83%) (Scheme 21)**.**[67]

3.2.2.3 Aminative dehalogenation of 4,6-dichloro-5-phenylpyrimidin-2-amine (83) with benzylamine

There are many ways to substitute the chlorine at position 4 with an amino group. However, this kind of reaction needs harsh conditions, which limits this to strongly nucleophilic alkylamines and anilines. A direct method for the aminative dehalogenation of **(83)** is by dissolving the chloropyrimidine in ethanol and introducing ammonia gas into the ethanolic solution at 0°C until saturation; the mixture is heated for five hours at 100°C. This method, however, is said to result in low yields, owing to the volatility of the ammonia.[67] Another method of using ammonia has been employed by Basford,[67] by heating the compound with aqueous ammonia in a sealed tube at 140-150°C for twelve hours. Because of the harsh conditions required and the low yield that have been reported for this method, the next step was to look at other procedures that could give the wanted amino group under mild conditions with reasonable yields. Basford[67] has also displaced one of the chlorines of **(83)** with the involatile arylamine; 4-chloroaniline at reflux in acetic acid for one hour.[67] This method inspired the idea of treating **(83)** with benzylamine under the same conditions, to introduce a 4-benzylamino group, which could later be debenzylated. Treatment of the dichloropyrimidine **(83)** with benzylamine gave **(86)** in 62% yield (Scheme 21). There was no evidence of formation of a 4,6-bis(benzylamino)pyrimidine, which shows that displacement of the second chlorine is more difficult.

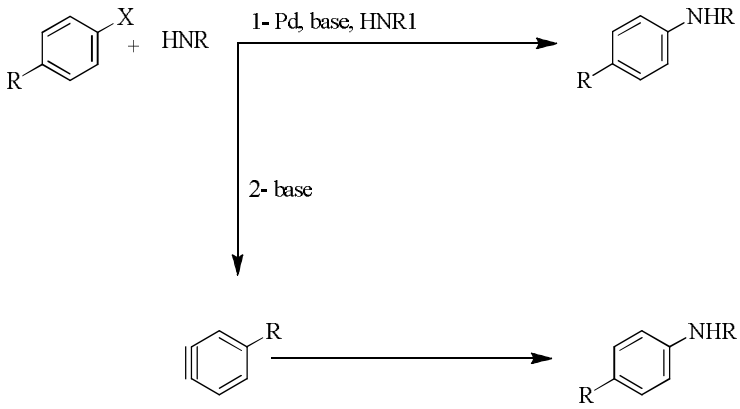
3.2.2.4 Amination of aryl halides

The traditional method for the conversion of an aryl halide to an aryl amine uses palladium, copper or nickel in presence of ligand. Palladium-catalysed reactions are of great interest in pharmaceutical and chemical synthetic research[68] (Scheme 22).



Scheme 22: Conversion of chloro to amino group by hydrogenation. Reagents and conditions: Pd_2dba_3, 1 mol %; ligand 4, 5 mol %; NaO*t*-Bu, 1.4 equiv; NH_3, 5 equiv; 0.042 M, 1,4-dioxane; 80 °C.

Amination of aryl halides can be done in the absence of a palladium catalyst. Generally, amination of aryl halides in the presence or absence of palladium catalyst, nucleophilic amine and appropriate base are essential for the reaction. Scheme 23 shows two pathways for the amination of aryl halides, both of them in presence of a nucleophilic amine and base.



Scheme 23: Amination of arylhalides with nucleophilic amine and base in presence and absence of Pd catalyst.

3.2.2.4.1 Attempts to remove the benzyl group of 4-benzylamino-6-chloro-5-phenylpyrimidine-2-amine (86)

In order to remove the benzyl group of **(86)**, it was heated with hydrogen bromide in acetic acid for two hours. NMR showed that there was no reaction but only the starting material remained. The reaction was therefore repeated overnight, for a longer time, but the result was the same.

The next trial used the most direct and common method for removal of a benzyl group. Hydrogenation of **(86)** with hydrogen/ palladium in methanol was carried out overnight

but this also failed to remove the benzyl group. . The ease of cleavage of benzyl protecting groups by hydrogenation correlates with the leaving group ability of the heteroatom attached to the CH_2. Thus benzyl esters and Cbz protecting groups are readily cleaved by hydrogenation (and with HBr), benzyl ethers are less reactive and benzylamines require forcing conditions (*e.g.* high pressure[69]or acidic solvent or transfer hydrogenation at high temperature[70]), if they react at all.

3.2.2.5 Aminative dehalogenation of 4,6-dichloro-5-phenylpyrimidin-2-amine (83) carrying a 4-methoxy benzyl (PMB)-protected amine instead of a benzyl amine

Clearly the protecting group needs to be more easily removed and 4-methoxybenzyl (PMB) was investigated next. This 4-methoxybenzyl (PMB) group should be removed more easily than benzyl by treatment with acid such as hydrogen bromide, other methods that could remove the 4-methoxybenzyl (PMB) group are hydrogenation and oxidation.[68] However, 4-methoxybenzylamine is slightly more nucleophilic than benzylamine and the reaction with **(83)** resulted in a mixture of the desired **(84)** (containing one 4-methoxybenzyl (PMB)-NH- group) and **(85)** in which both chlorines have been substituted. These were successfully separated by column chromatography to give **(84)** in 68% yield (Scheme 21).

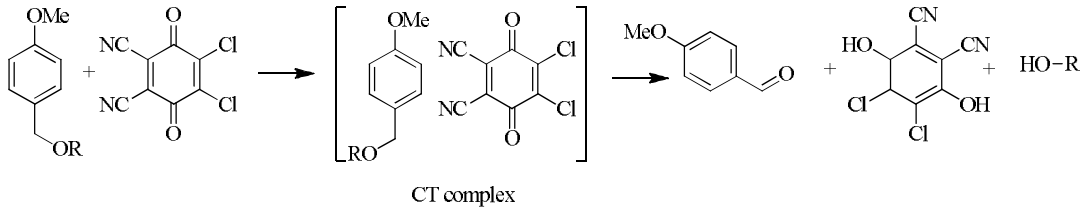
3.2.2.6 Cleavage of 4-methoxy benzyl (PMB) group of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84)

Hydrogenolysis with palladium is also known as a good method for the 4-methoxy benzyl (PMB) cleavage. Other reductive methods using reducing agents such as sodium cyanoborohydride.[71] may also be effective, but have been quite rarely studied.

Another way is treating the 4-methoxy benzyl (PMB) compound with an acid such as acetic acid in high temperature, trifluoroacetic acid in dichloromethane, or by using Lewis acids such as aluminium chloride or boron trifluoride.[71]

The third method is the oxidation method. The common oxidative agents used in this kind of reaction are 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and ceric ammonium nitrate (CAN) under neutral conditions. Oxidation by 2,3-dichloro-5,6-

dicyano-1,4-benzoquinone (DDQ) should normally happen in dichloromethane with small amounts of water or methanol. Adding water or methanol to the solvent system helps with the solubility, as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) is insoluble in dichloromethane. The reaction forms a charge-transfer complex between the electron donating ring of the 4-methoxybenzyl (PMB) and the electron accepting 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) resulting in formation of *p*-methoxybenzaldehyde (Scheme 24).[71]



Scheme 24: Oxidation mechanism by DDQ.

3.2.2.7 Attempts to remove the 4-methoxy benzyl (PMB) group of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84)

In order to remove the 4-methoxy benzyl (PMB) group, many attempts were made to test which method is most effective to cleave the PMB group of **(84)**, after replacing the chloro atom in position 6 with the amino derivatives.

Attempt 1: Removal of 4-methoxy benzyl (PMB) of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84) by acid

The first attempt was by treating compound **(84)** with hydrogen bromide in acetic acid under reflux. Even though the colour had changed to red in 5 hours, thin layer chromatography (TLC) and NMR showed that no reaction happened.

Another trial was with another acid, the reaction of **(84)** with sulfuric acid for 16 hours. This reaction also failed to remove the 4-methoxy benzyl (PMB) group and the starting material was recovered by aqueous work up with ethyl acetate and water.

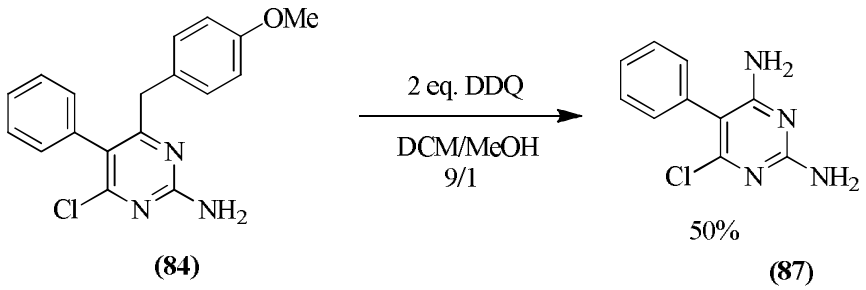
Attempt 2: Removal of 4-methoxy benzyl (PMB) of (84) by hydrogenation

Another attempt for 4-methoxy benzyl (PMB) removal was by transfer hydrogenation. This reaction was catalysed by palladium on carbon and ammonium formate in methanol, refluxed under argon. Thin layer chromatography (TLC) again showed that no reaction happened. The reaction was left then for a longer time, for another sixteen hours, thin layer chromatography (TLC) was the same with no trace of any reaction. The reaction mixture was filtered through Celite® and washed with methanol. NMR showed the recovered starting material **(84).**

Attempt 3: Removal of 4-methoxy benzyl (PMB) of (84) by oxidation

An oxidation with ceric ammonium nitrate (CAN) to remove the 4-methoxy benzyl (PMB) group was investigated. Compound **(86)** with ceric ammonium nitrate (CAN) in a solvent system 3:1 (acetonitrile /water), (to ensure the solubility), were mixed together at room temperature, following the literature,[71] but unfortunately the reaction failed.

Another oxidising agent investigated was 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ). Compound **(84)** was treated with one equivalent of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in a dichloromethane / methanol solvent system at room temperature for two days, changing the colour of the reaction mixture from green to partially red, thin layer chromatography (TLC) showed that a reaction had happened. The NMR spectrum showed that the reaction had gone half-way, with the 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) only being reduced to the semiquinone radical or quinone / hydroquinone charge-transfer complex. Thus an extra equivalent of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was added and the reaction was left for another day. The colour of the reaction mixture changed completely to red. NMR showed that the wanted compound **(87)** formed. The oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) successfully removed the 4-methoxy benzyl (PMB) group. Washing **(87)** with diethyl ether gave a reasonable yield of 50% (Scheme 25). This approach was the plan for the 4-methoxy benzyl (PMB) removal after replacing the chlorine atom in position 6 of **(84)** (Scheme 25)**.**



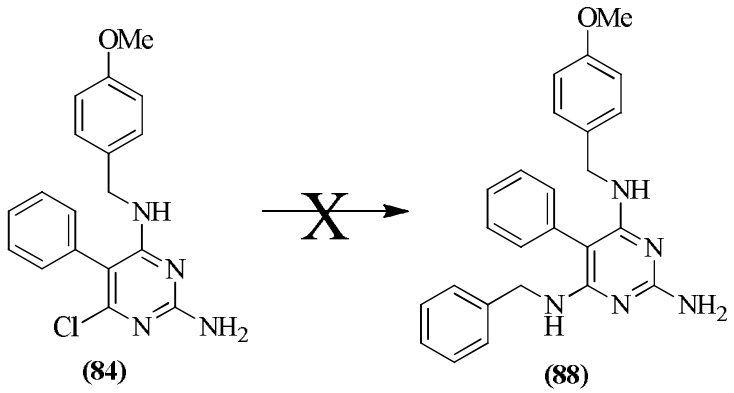
(84) **(87)**

Scheme 25: Removal of PMB by oxidation with 2 eq. DDQ.

3.2.2.8 Replacement of chlorine atom at position 6 of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84) with aromatic amine

For compound **(84)**, replacement of the chlorine atom at position 6 was very challenging, the main idea is to replace the chlorine atom with an amine, and this should be done by treating the compound **(84)** with benzyl amine, followed by a removal step for the benzyl group. Then, the removal of the 4-methoxy benzyl (PMB) group at position 4, which had been investigated, followed with the 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation. The idea of removing the 4-methoxy benzyl (PMB) group and the benzyl amine in one step would save time.

Because of the electrophilicity of the compound **(84),** with only one halogen atom at position 6 is decreased, so the reaction with an amine such as benzyl amine **(88)** will need a forcing condition and longer time. The compound **(84)** was treated with benzylamine and acetic acid under reflux at 130°C. The reaction was monitored and checked by thin layer chromatography (TLC) every three hours, but unfortunately no reaction happened. The next day extra equivalents of the benzyl amine were added but the result was the same (Scheme 26).



Scheme 26: Reaction of 6-chloro-4-(4-methoxybenzylamino)-5-phenyl-pyrimidine-2-amine (84) with benzyl amine. Reagents and conditions: $PhCH_2NH_2$,CH_3CO_2H, reflux at 130°C, 24 h.

3.2.2.9 Replacement of chlorine atom at position 6 of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84) with aliphatic amine instead of aromatic amine

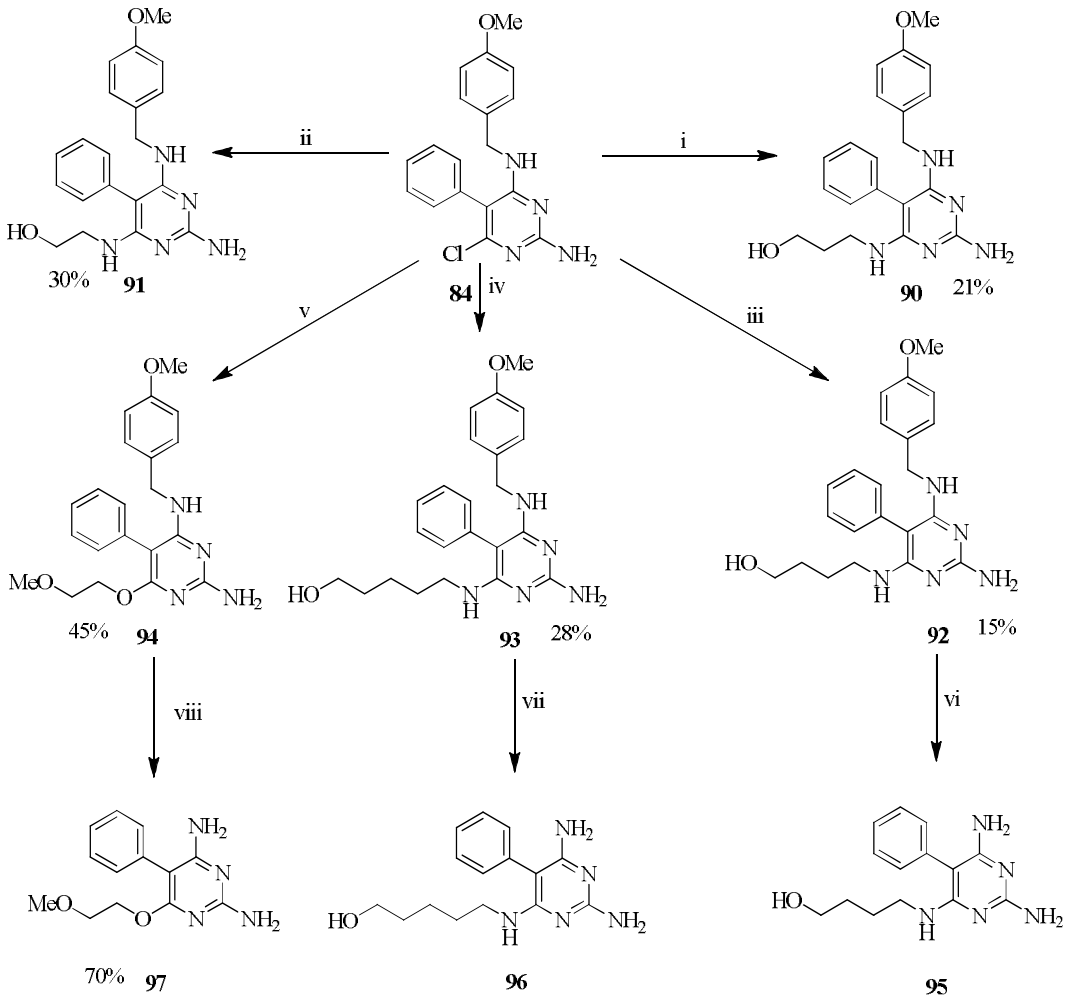
The target compounds are pyrimidine-2,4,6-triamines, so the 4-(4-methoxybenzyl)amino group and the 6-(hydroxyalkylamino) group could be introduced in either of two orders. Changing the order of reaction, the 4-(4-methoxybenzyl)amino group was introduced by reaction of the dichloropyrimidine with 4-methoxybenzylamine, The second chlorine is more difficult to displace (owing to the mesomeric electron-donating properties of the 4-PMBNH- group), so selective reaction is possible. Now the latter chlorine (in intermediate **(84)**) was displaced under forcing conditions with a series of aminoalcohols; in these the amine is much more nucleophilic than the alcohol and protection of the alcohol was unnecessary.

3.2.2.10 Dehalogenation of chlorine at position 6 of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84) with 4-aminobutanol

Changing the plan to replacement of the chlorine at position 6 of compound **(84)** with aliphatic amine, the selected amino alcohol that was first investigated was 4-aminobutanol.

Several attempts have been made to investigate the reaction with 4-aminobutanol. First, compound **(84)** was treated with 4-aminobutanol in ethanol in the presence of the base potassium carbonate at 78°C, overnight, no reaction occured.

The same problem with the poor electrophilicity of compound **(84)** had come to mind. Changing the reaction conditions with 4-aminobutanol was the next plan by repeating the reaction of **(84)** and 4-aminobutanol in the presence of potassium carbonate in different solvents with a higher boiling point. The reaction was done in 2-methoxyethanol at 130°C. Thin layer chromatography (TLC) showed a new spot in two days; the NMR spectrum of the crude mixture showed new compound was formed. However, an aqueous work-up and NMR spectroscopy revealed that the 2-methoxyethanol had reacted as the nucleophile to displace the chlorine, forming compound **(94)** (Scheme 27). This was shown by the presence of a singlet for the methoxy group in the spectrum at δ 3.22.

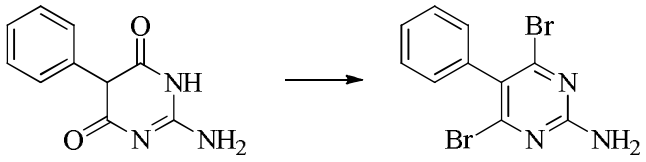


Scheme 27: Reactions with aminoalcohols(90-94) and deprotection of PMB groups (95-97). Reagents and conditions: i: $HO(CH_2)_3NH$, K_2CO_3, 150°C, 3d; ii: $HO(CH_2)_2NH$, K_2CO_3, 150°C, 3 d; iii: $HO(CH_2)_4NH$, K_2CO_3, 150°C, 3 d; iv: $HO(CH_2)_5NH$, K_2CO_3, 150°C, 3d; v: K_2CO_3, $CH_3O(CH_2)_2OH$, 130°C, 3 d; vi, vii, viii: DDQ, (DCM/MeOH)(9/1).

3.2.2.11 Testing the 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation to remove the 4-methoxy benzyl (PMB) after the methoxyethanol reaction (94)

Compound **(94)** was treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in dichloromethane / methanol mixture; the 4-methoxybenzyl (PMB) group was successfully removed to give **(97)** in a good yield. This was a proof that removal of PMB by DDQ oxidation is the ideal method.

3.2.2.12 Bromination of carbonyl group of 2-amino-5-phenyltetrahydropyrimidine-4,6-dione / 2-amino-5-phenylpyrimidine-2,4-diol (81) with PBr_3



(81) **(89)**

Scheme 28: Bromination of carbonyl group of 2-amino-5-phenyltetra-hydropyrimidine-4,6-dione.

The reaction of compound **(84)** with 4-aminobutanol was difficult. Changing the solvents was not helpful with the reaction, so replacement of the chloro atom with a bromo was adopted as the new plan. Bromine is a better leaving group, which should ease the reaction at milder conditions in a shorter time as well. The first attempt to brominate the carbonyl group of compound **(81)** with phosphorus oxybromide failed, so changing the brominating reagent was investigated. The chosen brominating agent was phosphorus tribromide. Dicarbonyl compound **(81)** was treated with phosphorus tribromide under reflux for sixteen hours to give the compound 4,6-dibromo-5-phenylpyrimidin-2-amine **(89)** (Scheme 28) in a very good yield of 86%.

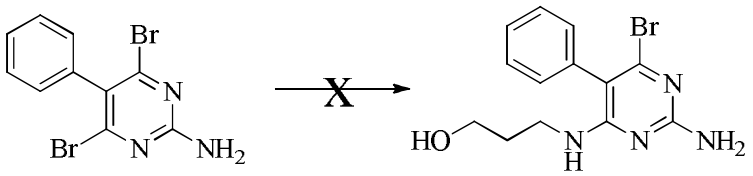
3.2.2.13 Reaction of 4,6-dibromo-5-phenylpyrimidin-2-amine (89) with 4-methoxybenzylamine

The replacement of the bromine atom at position 4 with 4-methoxybenzylamine used the same method with the dichloro compound **(83)** in acetic acid and 4-methoxybenzylamine. As the dibromo compound was expected to be much more reactive, the reaction was conducted at room temperature for two hours. However, no reaction occurred under these conditions, as shown by TLC. The temperature was raised in a stepwise manner. When no reaction appeared on TLC, heat was applied. The reaction mixture was heated at 40°C, but no reaction happened. Monitoring was continued every two hours with increasing temperature, first at 80°C, then at 120°C without any benefit. The NMR showed unidentified compounds, which meant that the starting material had decomposed at higher temperatures. The same experiment was

repeated again at lower temperature of 40°C for a longer time, but unfortunately no reaction happened and the starting material was recovered by column chromatography.

3.2.2.14 Reaction of 4,6-dibromo-5-phenylpyrimidin-2-amine (89) with 4-aminobutanol

Another attempt of 4,6-dibromo-5-phenylpyrimidin-2-amine **(89)** amination was trying the reaction of **(89)** with 4-aminobutanol instead of the 4-methoxybenzylamine. Compound **(89**) was treated with 4-aminobutanol in dry dimethylformamide (DMF) with molecular sieves but without base under reflux at 140°C (Scheme 29). No reaction occurred within twenty four hours. Sodium iodide was added as a nucleophilic catalyst but this did not aid the reaction. The same experiment of reaction **(89)** with 4-aminobutanol was repeated with adding one equivalent of potassium carbonate as a base but it failed as well.



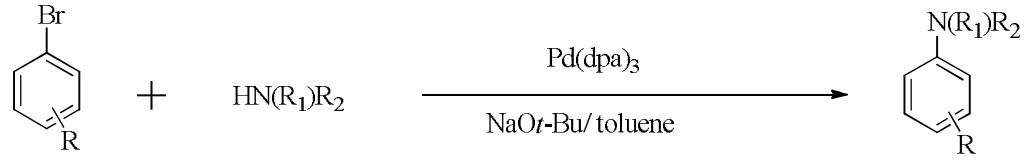
89

Scheme 29: Reaction of 4,6-dibromo-5-phenylpyrimidin-2-amine (89) with 4-aminobutanol. Reagents and conditions: $HO(CH_2)4NH$, DMF, 140° C, 24h.

3.2.2.15 Buchwald reaction

After these attempts to replace the bromine atom of compound **(89)** with amino compounds failed by different nucleophilic displacement, a new plan was introduced: investigating Buchwald palladium-catalysed reaction for aminative dehalogenations.

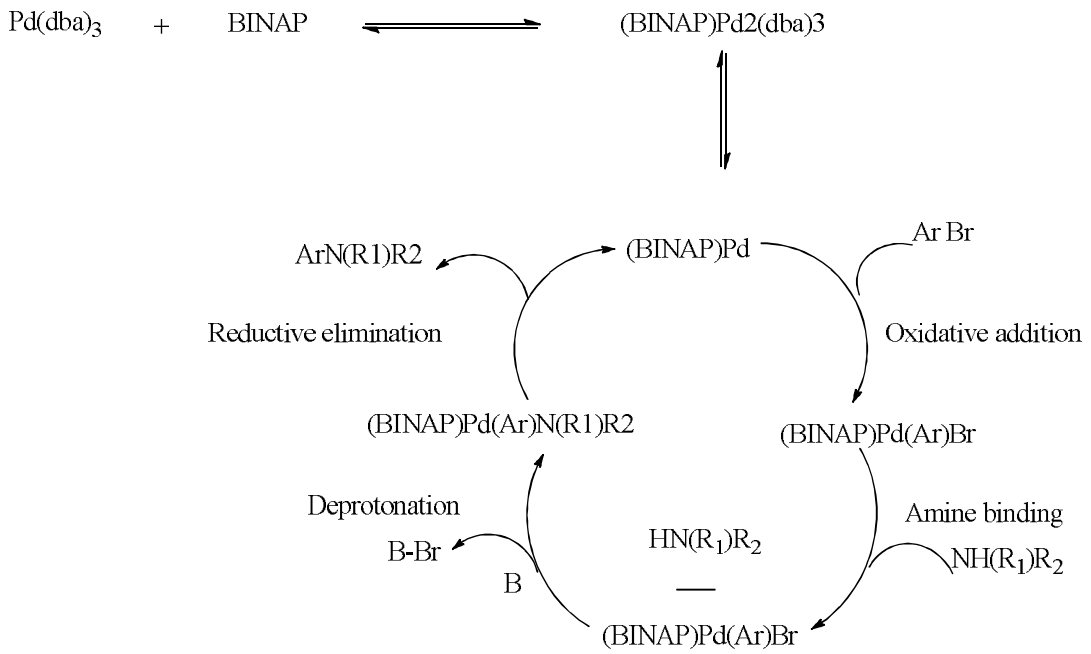
An example of palladium-catalysed aryl amine coupling with aryl halide, was reported by Buchwald *et al*,[72] is in presence of $Pd_2(dpa)_3$ and sodium *tert*-butoxide in toluene (Scheme 30).[72]



Scheme 30: Pd catalysed reaction of arylbromide in presence of NaO*t*-Bu and toluene.
R_1= alkyl, aryl. R_2= alkyl, aryl, H.

Another palladium catalysed reaction was introduced by Hartwig[68] but this method include using Josiphos-ligated palladium complex. He investigated a palladium catalytic system for aniline synthesis from the combination of 1.0 mol% palladium$_2$(dpa)$_3$ and 5.0 mol% of biaryl phosphine ligand, five equivalents of ammonia and sodium *tert*-butoxide (1.4 equiv) at 80°C in a sealed tube; dioxane as solvent gave the highest yield.[68] Application of Buchwald reaction on presence of ligand was the new plan for the project.

The mechanism of the Buchwald reaction (Scheme 31) consists of multiple steps; the overall process is the cross-coupling. Presence of palladium and supportive ligand will enhance the cross-coupling , then an oxidative addition of an aryl halide (Ar-X) to the ligand and the palladium, then the nucleophilic amine is added to oxidative addition complex. Because of the presence of the base, deprotonation of the complex will lead to a reductive elimination (Scheme 30).[73] Many studies about coupling of amines with aryl bromide have been carried out. 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) has shown effectiveness in this kind of reaction,[72] which was investigated during the project.



Scheme 31: Pd catalysed coupling reaction in presence of BINAP ligand.

Palladium-catalysed reactions have been an important subject in chemical synthesis, with the introduction of the supportive ligands such as 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) and xantphos. The palladium-catalysed reaction has developed and improved, especially with carbon-nitrogen coupling.[73]

The most important factors that influence palladium-catalysed reactions are: the palladium source, which will affect the formation of the catalysts and its effect on the reaction. The studies have shown that the most effective palladium catalyst is tris-(dibenzylidenacetone)dipalladium $Pd_2(dba)_3$ (which was the chosen palladium in our investigation) and palladium acetate $Pd(OAc)_2$, especially for aryl bromides.[73]

The second factor is the ligand used in the reaction, which always depends on the aryl halide substrate. Many studies have investigated different kinds of ligands; 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) showed a remarkable effectiveness for carbon-nitrogen coupling.[73]

The third factor is the aryl halide substrates and the nucleophilic amines in terms of their electrophilicity or nucleophilicity; these factors will also affect the rate of every

step on this reaction such as oxidative addition, amine binding to the formed complex and the deprotonation by the appropriate base.[73]

The fourth factor is the appropriate solvent. Choosing the appropriate solvent depends on the solubility of the substrates and the base. The most common solvents that are used in the palladium-catalysed reactions are toluene and dioxane; sometimes tetrahydrofuran (THF) and dimethylformamide (DMF), they could be used as well.[73]

The fifth factor is the temperature applied to the reaction; temperature determines the rate of the cross-coupling process.[73]

The last factor is the base. Strong bases such as sodium *tert*-butoxide could be used at room temperature; however, in some reactions, strong bases participate in unwanted side-reactions especially with some electrophilic and heterocyclic aromatics. Weak bases are also efficient in this kind of reaction and could be used at higher temperatures. The most common weak base that is used in the palladium-catalysed reactions is caesium carbonate. Caesium carbonate is mostly used in aminations with 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP); another weak base also useful in this reaction is potassium carbonate. The appropriate base also will affect the rate of every step on the reaction. The sensitivity of the functional groups of the aryl halide and the amines against the base should also be considered.[73]

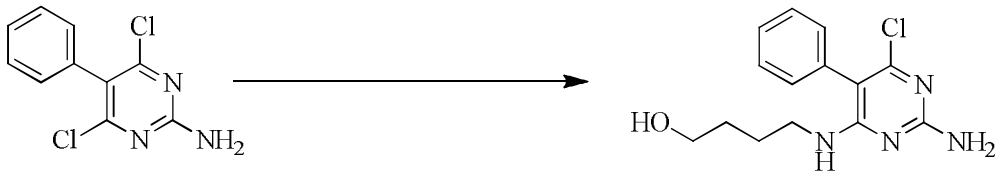
From these studies, the idea was to try a Buchwald reaction to replace the bromine atom of compound **(89)** with 4-aminobutanol and 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) ligand to fasten the reaction. Compound **(89)** and 4-aminobutanol were added to $Pd_2(dpa)_3$ and caesium carbonate in dioxane in the presence of BINAP at 110°C but there was no displacement of any of the bromine atoms.

After these trials with the dibromine **(89)** (Scheme 28) the reason for going back to the previous strategy with the dichlorine **(84)** (Scheme 27)**,** was as the chlorine atom is highly electronegative, this will lower the electron density at the site of the reaction with the nucleophile and lead to reduced side reactions.

3.2.2.16 Reaction of 4,6-dichloro-5-phenylpyrimidin-2-amine (83) with 4-aminobutanol

As the reaction of compound **(83)** with 4-methoxybenzylamine was successful, a new plan was introduced and investigated, which is the reaction of compound **(83)** with 4-aminobutanol first instead of 4-methoxybenzylamine.

Compound **(83)** reacted with 4-aminobutanol and potassium carbonate as a base in ethanol for 16 hours; careful control of the conditions allowed replacement of only one chlorine atom to give compound **(98)** (Scheme 32) in 55% yield.

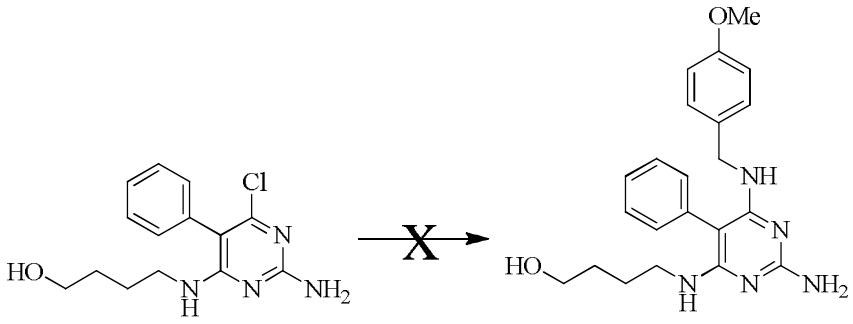


(83) **(98)**

Scheme 32: Reaction of compound (83) with 4-aminobutanol and K_2CO_3 in EtOH. Reagents and conditions: $HO(CH_2)_4NH$, K_2CO_3 in EtOH, 60°C, 16 h.

3.2.2.17 Reaction of 4-((2-amino-6-chloro-5-phenylpyrimidine-4-yl)amino)butanol (98) with 4-methoxybenzylamine in different solvents (investigating the opposite way)

One chlorine atom of compound **(83)** was replaced with 4-methoxybenzylamine (**84**) and then another experiment was conducted to replace this chlorine atom of compound **(83)** with 4-aminobutanol **(98)**. The reversal of the sequence of reactions was studied. Compound **(98)** was treated with 4-methoxybenzylamine and acetic acid at 130°C for two days, and no reactions occurred. Three sets of reactions were repeated and monitored for the same reaction again but in different solvents. The first one was in 2-methoxyethanol, the second one was in dimethylformamide (DMF), and the third was without solvent. None of these conditions worked to give the compound 4-(4-hydroxybutylamino)-6-(4-methoxybenzylamino)-5-phenylpyrimidin-2-amine **(99).** These experiments were demonstrated before realizing the reactivity of the methoxyethanol with the formation of **(94)** (Scheme 33)**.**



(98) (99)

Scheme 33: Attempted reaction of compound **(98)** with 4-methoxybenzylamine and acetic acid. Reagents and conditions: CH_3O_2CH, PMB, 130°C.

3.2.2.18 Whether to go with the synthesis of (84) or (98)?

As there still was no progress with replacement of the other chlorine atom by 4-aminobuatnol with both **(84)** and **(98)**. Further trials could continue with either **(84)** or **(98).** Formation of compound **(84)** by introducing the 4-methoxybenzylamino group first is much easier and quicker, as the reaction only takes two hours and the purification by column chromatography is much easier.

3.2.2.19 Reactions of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84) with different aminoalcohols

So continuing with a new plan for the reaction with **(84),** four compounds of aminoalcohols with different carbon length were used.

3.2.2.19.1 Reaction of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84) with different aminoalcohols in dry dimethylformamide (DMF)

Four sets of reactions were investigated; compound **(84)** was treated with one equivalent of 5-aminopentanol, 2-aminoethanol, 3-aminopropanol and 4-aminobutanol in dry dimethylformamide (DMF) under N_2 at 130°C. The reactions were continuously monitored by TLC then it was left over night. The next day, TLCs of these four reactions were checked and showed no reactions, four more equivalents of the

aminoalcohols were added to the four reactions and heating continued for more two days. No reactions happened; compound **(84)** was recovered by the aqueous work ups of the four reaction mixtures.

3.2.2.19.2 Reactions of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84) with different aminoalcohols and potassium carbonate in dry dimethylformamide (DMF) 'Method 1'

The same sets of experiments for compound **(84)** with the different aminoalcohols were also repeated but this time a base was added to the reactions. Two equivalents of each aminoalcohol and potassium carbonate were heated in dry dimethylformamide (DMF) at 130°C over night. The next day, TLC indicated no reaction, so four more equivalents of aminoalcohols and potassium carbonate were added to each reaction mixture; these were heated at 130°C for two days. At this point, TLC indicated some reaction but it did not continue through completion.

The four reactions were repeated under the same conditions but in a concentrated mixture with a small amount of dry dimethylformamide (DMF) to accelerate the reactions. Compounds **(90), (91), (94)** and **(93)** (Scheme 27) were successfully formed and confirmed by mass spectrometry. Purification of the four reaction mixtures was very difficult, as the excess of the aminoalcohols was difficult to remove from the formed compounds. Different methods were applied for more purifications, which were: several repeated column chromatography for each compound, washing the formed compounds with diethyl ether, and finally the normal work up with ethyl acetate/water have been done as well but without any benefit. These reactions gave low yields (varying from 10 to 20%). Moreover, these compounds were not completely pure because of the excess of aminoalcohols.

To solve the purifications problems for these reactions, the same sets of reactions were repeated with smaller amounts of aminoalcohols and the base. However, despite the use of only three equivalents of each aminoalcohol and potassium carbonate in very small amounts of dry dimethylformamide (DMF) under the same conditions, the results were the same and impure products were obtained. The same purification issues have not

been solved; these impurities are hard to get rid off maybe because of the high polarity of the aminoalcohols, and this problem gave an idea that the solvent might be the problem.

3.2.2.19.3 Reactions of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (88) with different aminoalcohols and potassium carbonate without solvent 'Method 2'

Reactions of compound **(84)** with the different aminoalcohols were repeated in the same conditions without solvents. The compounds were successfully formed, the purification by column chromatography was much easier, as the mixtures from the reactions were cleaner. These gave moderate yields (20 to 30%) but the products were purer compounds, especially with 4-aminobutanol **(92)** and 5-aminopentanol **(93)** with longer carbon chains, while **(90)** and **(91)** were still more difficult to purify, requiring several chromatography columns (Scheme 27).

So the displacement of the chlorine has been shown to occur using only three equivalents of aminoalcohols as nucleophiles and solvents, in the presence of potassium carbonate as a base, although these reactions still need optimisation processes.

3.2.2.20 Removal of 4-methoxy benzyl (PMB) protecting groups from compound (92) and (93) by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation

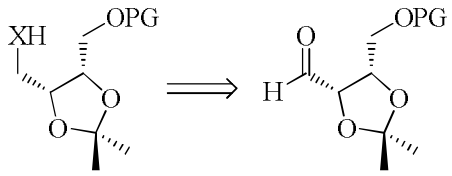
As removal of 4-methoxy benzyl (PMB) group has been investigated earlier in this project and was successfully applied, removal of the 4-methoxy benzyl (PMB) groups from **(92)** and **(93)** was also investigated by applying the same conditions**.**

Reaction of **(92**) and (**93)** with two equivalents of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) dichloromethane / methanol mixture for three days gave **(95)** and **(96)** in low yields, alongside impurities. thin layer chromatography (TLC) and column chromatography were difficult owing to the very polar nature of the target compounds. The compounds were identifiable by NMR and mass spectrometry. The reactions were repeated again at the same conditions and left for two weeks with the same results (Scheme 27).

3.2.3 Attempted synthesis of trihydroxy side chain:

Another plan also investigated in this project at the same time was the synthesis of the two building blocks: 5-phenyl-2,4-diamniopyrimidine motif and the trihydroxy side chain. The original idea came from El-Hamamsy's study.

The synthesis of the trihydroxyalkyl side-chain at position 6 of the pyrimidine ring can be achieved *via* different methods. Retrosynthetic analysis shows that these could be obtained from the aldehyde **(101)**. A similar protected aldehyde had been used by El-Hamamsy *et al.*[45] However, a different protecting group PG was required to make the reaction easier (Scheme 34), as Hamamsy had experienced some difficulties in introducing and removing the OBn protection under mild conditions.



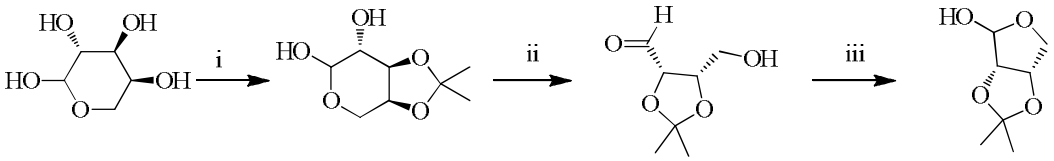
(100) **(101)**

Scheme 34: Structure of target protected triols (100) and retrosynthesis. X = O, NH, S.

El-Hamamsy *et al.*[45] had investigated both *cis*- and *trans*-substituted 4,5-dihydro-1, 3-dioxoles to achieve the synthesis of their diastereomeric products. For the *trans* series, the synthesis started with a diester (diethyl tartrate) **(21),** which is commercially available enantiomerically pure. The dihydroxy group of this ester reacted with 2, 2-dimethoxypropane under acid catalysis to give the acetonide **(22).** The ester functional groups were then reduced with lithium aluminium hydride to give the C_2-symmetric diol **(23).** The monoprotection of one hydroxyl group and oxidation of the other **(24)** gave the required aldehyde (Scheme 6).

The corresponding *cis* acetonide could not be approached from the *meso* diethyl tartrate because the intermediate diol would not have been C_2 symmetric and would have required chiral discrimination in the benzylation. Since this procedure is not practicable,

it was much easier to start with a sugar. The trihydroxyalkyl side-chain can be derived, in principle, from *D*-erythrose. In the literature,[74] the *D*-erythrose 2,3-acetonide **(105)** is reported to be synthesised by condensation of *L*-arabinose **(102)** with 2,2-dimethoxypropane and *p*-toluenesulfonic acid to give **(103)**, followed by cleavage of the diol with sodium periodate to give the aldehyde **(104).** A side-product of this reaction is formic acid, which catalyses the intramolecular cyclisation of the aldehyde to give the desired erythrose **(105)** (Scheme 35).[74] The ring-opened intermediate has the two exocyclic carbons differentiated as aldehyde and primary alcohol but, unfortunately, the equilibrium lies completely in favour of the cyclised form.



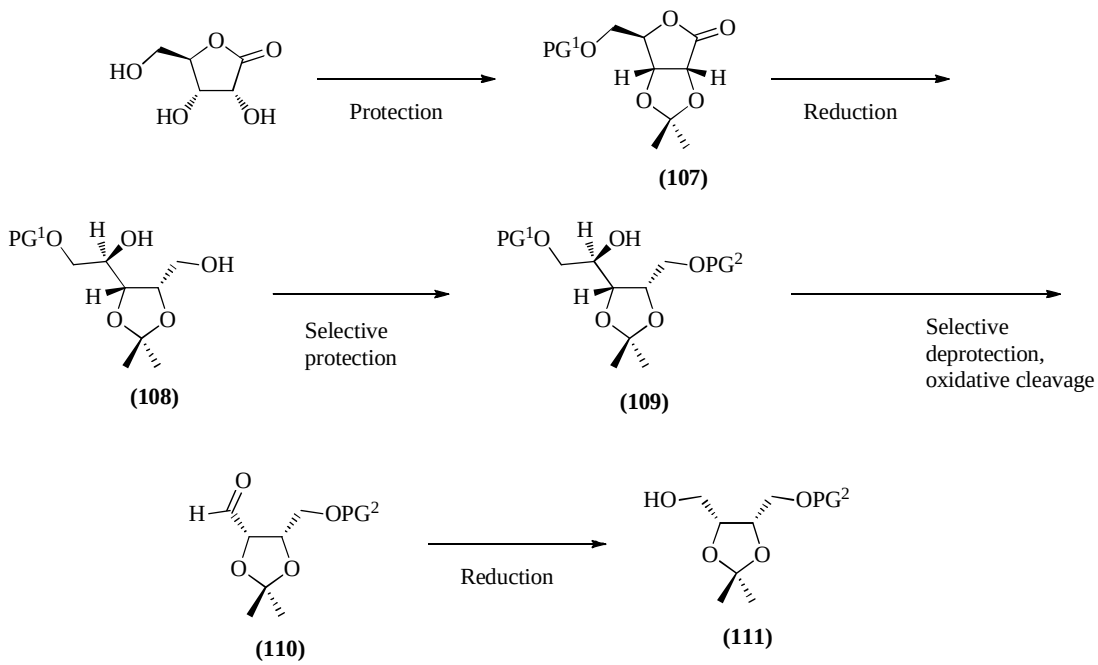
(102) **(103)** **(104)** **(105)**

Scheme 35: Literature synthesis of cis –acetonide. Reagents and conditions: i, DMP, TsOH; ii, $NaIO_4$; iii, H^+.

3.2.3.1 Synthesis of the trihydroxy side chain starting with the ribonolactone

The plan was therefore changed to start with a pentose such as *D*-(+)-ribonolactone **(106),** which is a commercially available lactone carrying three hydroxy groups, plus one masked as the lactone. These hydroxyl groups require protection to avoid unwanted reactions.

Scheme 36 explains the new pathway, which starts with protection of the vicinal hydroxyl groups of ribonolactone as the acetonide. The primary hydroxyl group is then protected with protecting group PG^1 to give the fully masked ribonolactone **(107)**. Reduction of the ester then reveals a primary alcohol and a secondary alcohol in **(108)**. These alcohols should then be discriminated by protection of the CH_2OH with PG^2 in **(109)**. Now PG^1 is removed to reveal a vicinal diol, which can be cleaved oxidatively to give the aldehyde **(110)**. Finally, the aldehyde is reduced to give the target intermediate **(111)**.



Scheme 36: New approach to synthesis of the trihydroxy side chain.

3.2.3.1.1. Acetonide protection of the hydroxyl groups of ribonolactone

The protection and the deprotection procedures of the anomeric centre of chiral carbohydrates such as pyranose and furanose, are difficult, because it is hard to predict the regioselectivity on the hydroxyl groups. That is why some carbohydrates have been involved in spectroscopic and crystallographic studies to understand the best conformation for different kinds of reactions.[75]

In practice, the 2,3-vicinal hydroxyl groups of ribonolactone can be protected by forming an acetal group. There are two common methods for acetonide protection of the vicinal groups. One is known as classical acetonation with acetone and sulfuric acid under high temperature for 24 hours. The other one is with 2,2-dimethoxypropane in dimethylformamide (DMF) and its advantages over the classical method are shorter time of reaction and no purification was needed.[76] The classical method for the acetonide formation with acetone and an acid catalyst such as hydrochloric acid or sulphuric acid under reflux is a thermodynamic control, and the other method with 2,2-dimethoxypropane (DMP) in dimethylformamide (DMF) is a thermokinetic. The

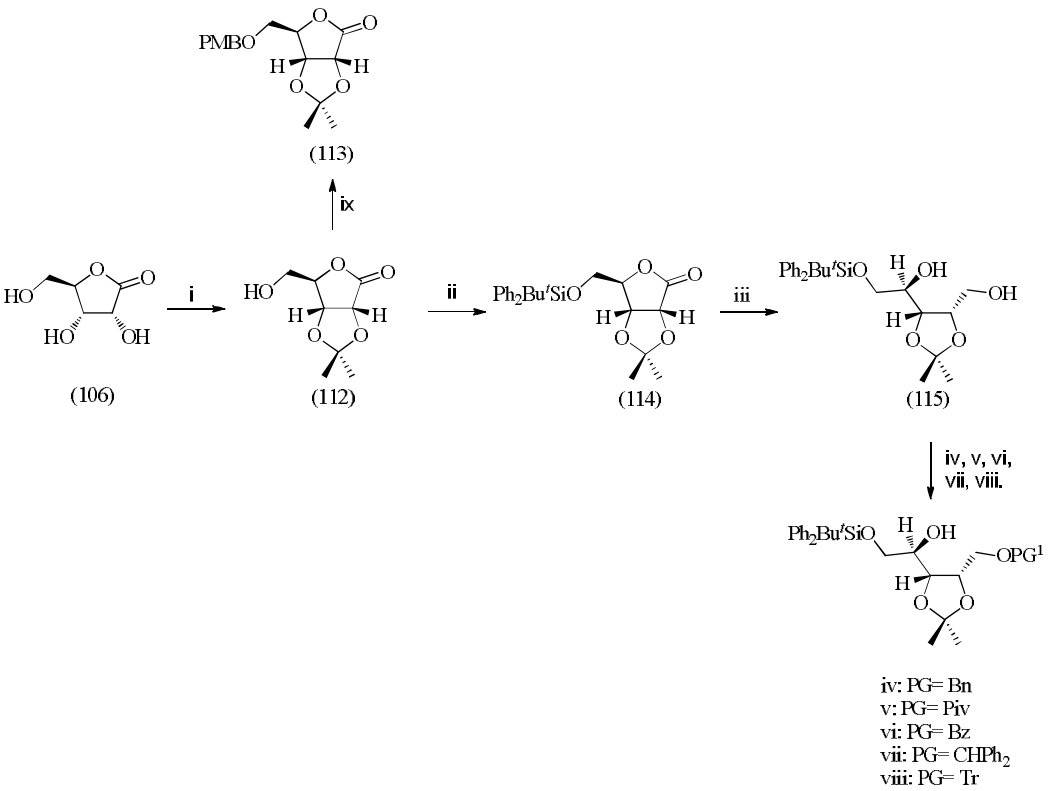
thermokinetic reaction with dimethoxypropane (DMP) also has two different ways that are all successful in high yields, one with dimethoxypropane (DMP) and *p*-toluenesulfonic acid, and the other one is dimethoxypropane (DMP) with pyridinium *p*-toluenesulfonate in dimethylformamide (DMF). Pyridinium *p*-toluenesulfonate is a weaker acid than *p*-toluenesulfonic acid and is useful when acid-sensitive functional groups are involved such as *tert*-butyldimethylsilylethers,[77] the acetal protecting group is considered a good choice for aldehydes and ketones because of its stability to base and the ease of its removal by acid. It is inert to nucleophilic reagents or aqueous base-catalysed reactions and also protects the hydroxyl groups against oxidation.

D-(+)-ribonolactone **(106)** was treated with 2,2-dimethoxypropane in the presence of pyridinium toluenesulfonate as acid catalyst to give ribonolactone monoacetonide[76] **(112)** in 62% yield (Scheme 36). ^{1}H NMR confirmed the presence of the two methyl groups of the acetonide upfield.

3.2.3.1.2 Protection of the primary hydroxyl group of the protected ribonolactone 3a*R*,6*R*,6a*R*-2,2-dimethyl-6-hydroxymethyldihydrofuro[3,4-*d*][1,3]dioxol-4(3*H*)-one (112)

3.2.3.1.2.1 Protection of with 4-methoxy benzyl (PMB) group

The next step was protecting the primary hydroxyl group of the protected ribonolactone 3a*R*,6*R*,6a*R*-2,2-dimethyl-6-hydroxymethyldihydrofuro[3,4-*d*][1,3]dioxol-4(3*H*)-one (112). Initially, the 4-methoxybenzyl (PMB) group was investigated. 4-methoxybenzyl (PMB) is able to survive various reaction conditions and is easily removed by treating the compound with acid or by catalytic hydrogenation. The 4-methoxybenzyl (PMB) group is very popular and much less stable to acid than benzyl ether.[77] Generation of the alkoxide with sodium hydride and reaction with 4-methoxybenzyl chloride[78] gave **(113)** (Scheme 36) at a low yield of 20%. The presence of the 4-methoxybenzyl (PMB) group was shown by ^{1}H NMR spectroscopy. Because of the low yield of the reaction and the sensitivity of the 4-methoxybenzyl chloride to moisture, alternative protecting groups (silyl ethers) were investigated.



Scheme 37: Synthesis of protected reduced sugars. Reagents and conditions: i: $Me_2C(OMe)_2$, pyridinium tosylate, 60°C, 4 h, 62%; ii: $Ph_2ButSiCl$, imidazole, DMF, 20°C, 24 h, 62%; iii: $NaBH_4$, EtOH, 2 h, 89%; iv: BnBr, NaH, DMF, 24 h; v: Me_3CCOCl, Et_3N, CH_2Cl_2, 24 h, 54%; vi: PhCOCl, Et_3N, CH_2Cl_2, 24 h; vii: Ph_2CHCl, NaH, DMF; viii: Ph_3CCl, NaH, DMF; ix: 4-MeOPhCH_2Cl, NaH, DMF, 24 h, 20%.

3.2.3.1.2.2 Protection with *tert*-butyldiphenylsilylether (TBDPS) (114)

Silyl ether groups include a silicon atom attached to oxygen. Their stabilities are different, and depends on the substituents attached to the silyl ether, which will affect their stability towards acid / base hydrolysis, oxidation, reduction and column chromatography. The reason for the stability for most silyl ethers, such as *tert*-butyldiphenylsilylether (TBDPS), is the presence of the bulky group that will increase the steric effect to give greater stability. Silyl ethers are easily formed from alcohols with the corresponding silyl chloride and base such as triethylamine. Also, its cleavage is easy, silyl ethers could be cleaved by fluoride ions. *tert*-Butyldiphenylsilylether (TBDPS) is the most stable to acid hydrolysis, reduction, under basic conditions because of its bulkiness; also it can be cleaved under acidic condition such as (acetic acid-tetrahydrofuran(THF)-H_2O) (3:1:1); *p*-toluenesulfonic acid in methanol and with hydrogen fluoride in acetonitrile or in pyridine.[77]

tert-Butyldiphenylsilyl (TBDPS) was chosen because of its relative stability against acid and base hydrolysis, oxidation and reduction. The primary alcohol **(112)** was treated with *tert*-butyldiphenylsilyl chloride *(*TBDPS) and imidazole.[79] Reaction of the *tert*-butyldiphenylsilyl chloride (TBDPS*)* with the imidazole gives (TBDPS*)*-imidazole as an intermediate, which is trapped by the primary hydroxyl group. Indeed, (TBDPS)-imidazole is selective for primary hydroxyl groups, owing to the steric bulk of the *tert*-butyl group and phenyl groups. The completely protected sugar **(114)** was obtained in 62% yield. The ^{1}H NMR showed the two phenyl groups of the *tert*-butyldiphenylsilyl (TBDPS) group at the aromatic region and a single peak of the *tert*-butyl at an upfield chemical shift (Scheme 37).

3.2.3.1.3 Reduction of carbonyl group of (3a*R*,6*R*,6a*R*)-6-((*tert*-Butyldiphenylsilyloxy)methyl)-2,2-dimethyldihydrofuro[3,4-*d*][1,3]dioxol-3(3a*H*)-one (114)

To reduce the lactone carbonyl group of compound **(114)**, various reagents were considered. The most common reducing agents for carbonyls are sodium borohydride, lithium borohydride and lithium aluminium hydride. Sodium borohydride would not normally reduce esters. Lithium borohydride is selective for reduction of esters, aldehydes and ketones but not amides and carboxylic acids.[80]

3.2.3.1.3.1 Reduction of (114) by lithium borohydride

The ester carbonyl group at position-3 of compound **(114)** was reduced to the diol by using the reactive reducing agent lithium borohydride to give **(115)** in 68% yield (Scheme 37), ^{1}H NMR showed the new hydroxy groups at δ 3.06.

3.2.3.1.3.2 Reduction of (114) by sodium borohydride

There are some factors that affect the reducing action of sodium borohydride, which are: the solvent, the catalyst and the activating substituents.[80] In the litreature,[81] a substituted ribonolactone with a silyl ether group was reduced by sodiumborohydride was reported. This milder reducing agent sodium borohydride will not usually reduce esters but only aldehydes and ketones. However, ester **(114)** was reduced to give (*R*)-2-(*tert*-

butyldiphenylsilyloxy)-1-((4*R*,5*S*)-5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)ethanol **(115)** with this reagent with a higher yield of 89%. This compound is a lactone with an electronegative oxygen atom at the α-position, which has an electron-withdrawing inductive effect making the carbonyl group more electrophilic and more susceptible to sodium borohydride reduction (Scheme 37).

3.2.3.1.4 Protection of the primary hydroxyl group of (*R*)-2-(*tert*-butyldiphenylsilyloxy)-1-((4*R*,5*S*)-5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)ethanol (115) attempts with different protecting groups

Attempt 1: Protection of the hydroxyl group of (115) with 4-methoxy benzyl (PMB)

The reduction of the carbonyl group gave two hydroxyl groups, one primary and one secondary. An attempt was made to protect the primary hydroxyl group selectively by treating compound **(115)** with 4-methoxybenzyl chloride and sodium hydride. However, this gave a product without the (TBDPS) group (Scheme 37).

Attempt 2: Protection of the hydroxyl group of (115) with benzyl ether

Another group for a hydroxyl protection is benzyl ether, they are stable to many aqueous acidic and basic conditions, and most reducing agents or mild oxidising agents at high temperatures do not affect them. Also it can be removed by catalytic hydrogenolysis, which is the mildest method; the catalyst of choice is palladium on charcoal or palladium hydroxide. Some methods of making benzyl ethers are alkylation of metal alkoxide with benzyl bromide or benzyl chloride; these are the most common methods, since the metal alkoxide is usually generated with sodium hydride or potassium hydride, but these methods are not compatible with base-sensitive functional groups. Another method of benzyl ether formation is by using benzyl bromide in the presence of silver oxide (Ag_2O), especially for substrates that do not survive the metal hydride.[77] Benzylation was also attempted and compound **(115)** (Scheme 37) was treated with benzyl bromide and sodium hydride in dry dimethylformamide (DMF).[78] NMR analysis again showed the same result as for the 4-methoxybenzyl (PMB)

reaction, with the *tert*-butyldiphenylsilyl (TBDPS) group again lost. The possible explanation for this problem might be the use of sodium hydride, which is a source of hydride that may attack the silicon atom causing cleavage of the *tert*-butyldiphenylsilyl (TBDPS) group. Alternatively, the alkoxide generated as an intermediate may promote loss of the (TBDPS) by neighboring-group participation.

Attempt 3: Protection of the hydroxy group of (115) with pivalate and benzoate esters

Since the alkoxide may be responsible, at least in part, for the loss of the *tert*-butyldiphenylsilylether (TBDPS) silyl protection, protection of the primary hydroxy group of **(115)** was investigated under conditions that do not generate alkoxides. Compound **(115)** was protected by the pivaloyl group, increased hydrolytic stability of the ester function is attained by pivalate, they are slower to deprotect than acetate and benzoate, and can be cleaved by reduction with lithium hydroxide in methanol or potassium carbonate in methanol under reflux.[77]

Protection was achieved by treating **(115)** with triethylamine, followed by heating with pivolyl chloride overnight to give an oil in 54% yield, after a difficult purification by column chromatography (Scheme 37). Unfortunately, there is no evidence of the formation of **(115).**

Another idea attempted was protection of this hydroxyl group with benzoate. It can be removed by tetrafluoroboric acid in methanol.[77] Compound **(115)** was treated with benzoyl chloride in pyridine at room temperature for twenty four hours and caused loss of the *tert*-butyldiphenylsilylether (TBDPS) group (Scheme 37).

Attempt 4: Protection of the hydroxyl group of (115) with trityl and diphenylmethyl groups

In another strategy, attempts were made to introduce triphenylmethyl (trityl, Tr) and diphenylmethyl protecting groups to the primary hydroxyl of **(115)**. Triphenylmethyl is a very bulky hydrophobic group, which is selective for introduction at primary alcohols.[77] This protection is stable to base, hydride-type reducing agents and

nucleophiles and can easily be removed by acidic conditions because of the stability of the triphenylmethyl carbocation. It can be removed with protic acids in methanol such as acetic acid; most Lewis acids such as zinc bromide in methanol or iron(III) chloride can also cleave it. Some have reported that 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in acetonitrile may cleave triphenylmethyl as well as liquid ammonia with Na, while formation of triphenylmethyl normally uses pyridine or 4-dimethylaminopyridine (DMAP) as solvents.[77] Another known method for the protection of the hydroxyl group with triphenylmethyl is by treating the hydroxy compound with chlorotriphenylmethane in pyridine for sixteen hours at 70°C.[76] Both of these methods for the triphenylmethyl formation would achieve their selectivity for the primary hydroxyl owing to their steric bulk. Triphenylmethyl ethers can be cleaved with mild acid or by hydrogenolysis, whereas diphenylmethyl ethers are usually cleaved by hydrogenolysis or by strong acid. Protection of the hydroxyl group with triphenylmethyl was attempted in sodium hydride and dimethylformamide (DMF) at room temperature for twenty four hours,[79] but no reaction happened. The protecting group was changed to a diphenylmethyl ether in sodium hydride and dimethylformamide (DMF) for fourty eight hours. Unfortunately, both reactions failed and the starting material was recovered by column chromatography (Scheme 37).

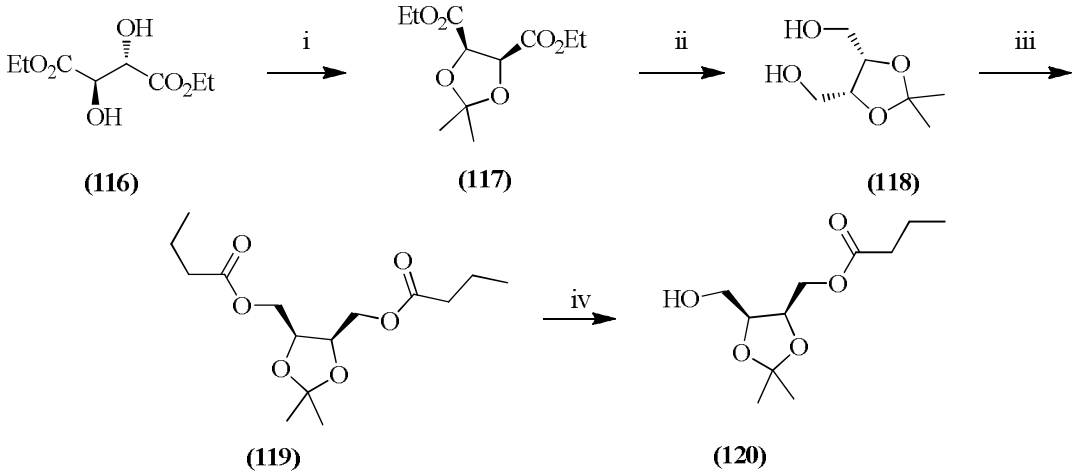
All these difficulties in the protection of the hydroxyl group in the presence of *tert*-butyldiphenylsilylether (TBDPS), which are supposed to be highly stable, led to thinking about different strategies that El-Hamamsy[45] (Scheme 6) had followed by using the *meso*-tartaric acid instead.

3.2.3.2 Synthesis of the trihydroxy side-chain starting with tartaric acid

The plan started with *meso*-tartaric acid, which is commercially available. Reaction of the diethyl ester from the tartaric acid and ethanol in the presence of pyridinium-*p*-toluenosulphonate (Scheme 38) gave the diester in 40% yield. The yield was raised to 58% by use of *p*-toluenesulfonic acid as the catalyst in the esterification reaction.

3.2.3.2.1 Protection of the diethyl tartrate to give (117)

Compound (**117)** was formed from the protection of the vicinal hydroxyl groups of **(116)**; acetonide formation was the best choice by following the kinetic control method with 2,2-dimethoxypropane in dichloromethane with *p*-toluenesulfonic acid to give the protected diethyl tartate[45] **(117)** in very low yield. Using a higher-boiling solvent raised the yield; boiling toluene was the solvent for the acetonide protection and that gave **(117)** in 86% yield (Scheme 38).



Scheme 38: Synthesis of the trihydroxy side chain from *meso*-tartaric acid. Reagents and conditions:i:TsOH, DMP ii: $LiAlH_4$; iii: Butanoylchloride, Et3N, DCM; iv: PCL, *i*-pr_2o.

3.2.3.2.2 Reduction of the diethyl ester on diethyl *R,R*-2,2-dimethyl-1,3-dioxolane -4,5-dicarboxylate (117)

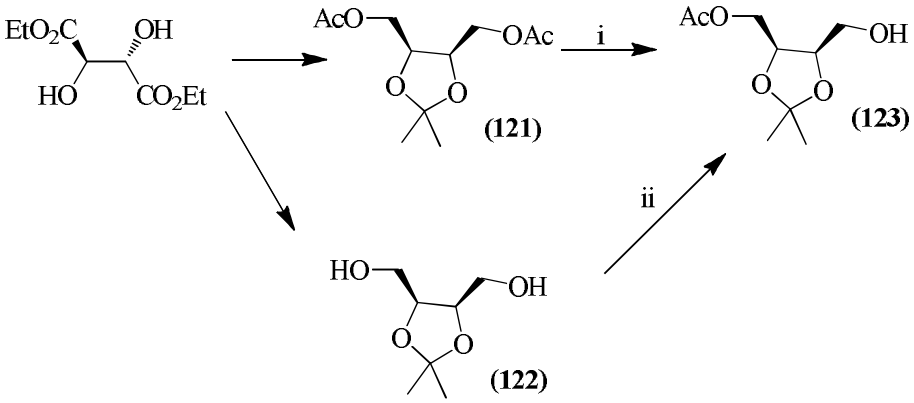
To reduce the two ethyl esters on compound **(117)**, many studies had reported that sodium aluminium hydride and lithium aluminium hydride have the same effect as reducing agents.[82] Treatment of **(117)** with lithium aluminium hydride in tetrahydrofuran (THF)[45] gave compound **(118)** in 55% yield (Scheme 38).

3.2.3.2.3 Protection of the diol of *S,S*-4,5-Di(hydroxymethyl)-2,2,dimethyl-1,3-dioxolane (118) with butanoate

In order to avoid the difficulties that El-Hamamsy[45] had with the benzyl protection of the diols **(118),** the idea of trying with an ester such as butanoate by reaction of **(118)** with butanoyl chloride in basic condition was investigated. Because the following step is to remove one of the stereoselective group in the *meso* compound, it might be applicable with the lipase enzyme. This protection was achieved successfully to give **(119)** in 65% yield (Scheme 38).

3.2.3.2.4 Attempted hydrolysis of the formed ester *S,S*-4,5-di(butanoyloxymethyl)-2,2,dimethyl-1,3-dioxolane (119) by lipase enzyme

Lipase is known as a hydrolytic agent for asymmetric synthesis. It can hydrolyse the prochiral diesters of diols to homochiral monoacetates. There was a study that investigated the lipase hydrolytic action from the commercially available *meso*-tartaric acid to the diol (**121**) and the diacetate (**122**). It was determined that *Pseudomonas cepacia* lipase (PCL) hydrolyses the diacetate to monoacetate in 81%, with higher enantioselectivity especially in an immiscible organic solvent such as diethylether, diisopropyl ether or hexane, and if it is in an emulsion (organic solvent/ water) will give 91% monoacetate. Also, it has been reported that the acetylation rate of (PCL) for a diol to a monoacetate is high as well giving a 91% yield at room temperature with acetic anhydride and toluene[83] (Scheme 39).



Scheme 39 : Lipase hydrolysis of the tartaric acid.i: PCL/ phosphate buffer, pH 7.0, *i*-Pr_2O/H_2O, rt, 5 h. ii: PCL, Ac_2O/ toluene, rt, 10 h.

In order to hydrolyse the ester to an alcohol (**122)** a lipase enzyme reaction was attempted. Compound **(119)** (Scheme 39) and PCL were stirred in the phosphate buffer and diisopropyl ether (*i*-Pr_2O). The reaction was monitored at pH 7 for five hours, the mixture was extracted with diethylether. However, there was no reaction as the starting material was recovered by column chromatography.[83]

4. Conclusion

This project has focused on the development of a novel selective inhibitor of *M. tuberculosis* DHFR, based on that which was reported in a previous study.[45] The target compounds all contain a 2,4-diaminopyrimidine moiety. The project has focused on exploring the DHFR binding site using a linker at position 6 containing either O or N.

The synthesis of N-*p*-methoxybenzylamino-2,4-diamino-5-phenylpyrimidine was carried out by condensation of diethylphenyl malonate with guanidine, followed by halogenations of the carbonyl groups to create dichloro or dibromo intermediates. The dichloro intermediate, as well as dibromo, were successfully formed after one attempt with the brominating agent, phosphorus oxybromide, for the dibromo intermediate. One of the chlorine atoms of the dichloro intermediate was then substituted with a 4-methoxybenzylamino group. This achieved compound was essential for both plans in the project. The two plans were applied after synthesizing 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine; the other chlorine atom was substituted with different aminoalcohols (amino butanol, pentanol, ethanol and propanol) after several attempts with aromatic amines. Investigating several conditions such as different solvents, the presence and absence of base, and the temperature have achieved the best reaction conditions to replace the chlorine atom with an amino alcohol especially with amino butanol and pentanol but in low yields. These conditions use three equivalents of potassium carbonate with three equivalents of the amino alcohol at 130°C without solvent. Then, the *p*-methoxybenzylamino group was successfully removed by DDQ oxidation. Achievement of the 2,4-diaminopyrimidine with the amino alcohol side chain at position 6 of the pyrimidine ring requires optimisation and several purifications by column chromatography. The Buchwald reaction has also been investigated with the dibromo intermediate to substitute one of the bromine atoms with aminobutanol in the presence of BINAP ligand, which has shown good results with aryl bromide in literature. Unfortunately, the substitution failed.

The second plan was synthesizing 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (the essential compound for both plans), followed by the

synthesis of the trihydroxy side chain, to link it to position 6 of the pyrimidine ring. Synthesis of the trihydroxy side chain, started from the ribonolactone, followed by acetonide protection of the vicinal hydroxyl groups. The investigation of different protecting groups for the primary hydroxyl group led to a protection with the *tert*-diphenyldimethylsilyl group because of its stability and bulkiness. This was followed by a reduction of the ester carbonyl group, and gave a good result with sodium borohydride, which normally does not reduce esters. The reduction of the ester resulted in the formation of primary and secondary hydroxyl groups. Protection of the primary hydroxyl group was investigated with different protecting groups, which unfortunately failed. Changing the synthetic strategy of the synthesis of the trihydroxy side chain showed good results in literature in the hydrolysis of esters, beginning with *meso*-tartaric acid, followed by acetonide protection of the vicinal hydroxyl groups, followed by reduction to the diol with lithium borohydride, and finally esterfication of the diols with butonoyl chloride to test the hydrolysis of one ester by the lipase enzyme. Unfortunately, it was not successful. For future work after the synthesis of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine with different amino alcohols, the following is needed:

- Optimisation of the reactions of 6-chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine with different amino alcohols (amino butanol, pentanol, ethanol and propanol), while finding a perfect way for purification.
- Removal of the PMB group by oxidation with DDQ.
- Testing of 4-(2-hydroxyalkyllamino)-6-(4-methoxybenzylamino)-5-phenylpyrimidin-2-amine against the mycobacterial DHFR.

Experimental

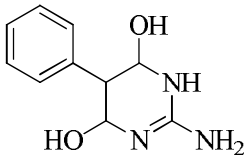
General experimental

Chemical reagents were purchased from Sigma, Aldrich, Fluka and Novabiochem. TLC was carried out on Merck aluminium-backed TLC plates Silicagel 60 F_{254} and viewed using UV light (λ = 254 nm). Column chromatography was carried on silica gel 60 (35 – 70 micron).

All reactions were carried out under a static atmosphere of nitrogen and stirred magnetically at room temperature unless otherwise stated. Solutions in organic solvents were dried with $MgSO_4$.

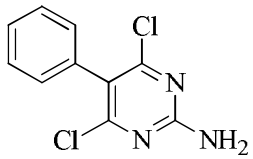
NMR data were obtained on a Varian Mercury VX spectrometer (400 MHz). The chemical shifts are recorded in parts per million (ppm) relative to tetramethylsilane. Mass spectrometry was carried out on a microTOF^{TM} from Bruker Daltonics (Bremen, Germany) using an electrospray source (ESI-TOF). Melting points were obtained using a Reichert-Jung heated-stage microscope.

2-Amino-5-phenyltetrahydropyrimidine-4,6-dione / 2-Amino-5-phenylpyrimidine-2,4-diol[67] (81)



NaH (2.26 g, 94.2 mmol) was added carefully to dry MeOH (60 mL). Guanidine hydrochloride (6.00 g, 62.8 mmol) and diethyl phenylmalonate **(80**) (11.6 g, 52.2 mmol) were added to this solution of NaOMe and the mixture was stirred under reflux for 3 h. The solvent was evaporated. The residue, in water, was stirred with decolourising charcoal for 15 min. The suspension was filtered. Acetic acid (20 mL) was added to the filtrate at 80°C then the mixture was filtered to give **(81)** (4.80 g, 46%) as a white solid: mp >350°C (lit.[84] mp 333-336°C decomp.); ^{1}H NMR ($CDCl_3$) δ 6.66 (2 H, br, 2 × OH), 7.00 (1 H, tt, *J* = 8, 1 Hz, Ph 4-H), 7.17 (2 H, t, *J* = 8 Hz, Ph 3,5-H_2), 7.53 (2 H, d, *J* = 8 Hz, Ph 2,6-H_2), 10.43 (2 H, br, NH_2); MS *m/z* 429.1273 (2 M + Na) ($C_{20}H_{18}N_6NaO_4$ requires 429.1257), 226.0576 $[M + Na]^+$ [$C_{10}H_9N_3NaO_2$ requires 226.0592].

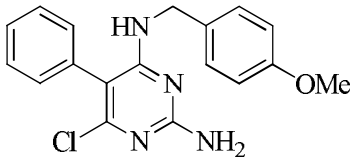
4,6-Dichloro-5-phenylpyrimidin-2-amine[67] (83)



Compound **(81)** (4.00 g, 19.7 mmol) and phosphorus oxychloride (18.0 mL, 119 mmol) were stirred under reflux for 3 h. Ice was added to the solution and the mixture was stirred for 2 h, then the mixture was neutralised with aq. ammonia (4.0 mL, 35%). The precipitate was filtered off and dried to give **(83)** (3.9 g, 83%) as a white solid: mp 285-

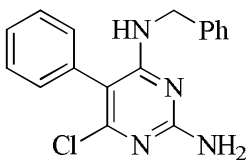
287°C (lit.[65] mp 221-222°C); ^{1}H NMR ($CDCl_3$) δ 5.25 (2 H, br, NH_2), 7.27-7.46 (5 H, m, Ar). ^{13}C NMR ($CDCl_3$) δ 118 (pyrimidine 5-C), 128.26 (Ph 4-C), 128.35 (Ph 3,5-C_2), 130.27 (Ph 2,6-C_2), 160.06 (pyrimidine 4,6-C_2), 161.25 (pyrimidine 2-C).

6-Chloro-4-(4-methoxybenzylamino)-5-phenylpyrimidine-2-amine (84)



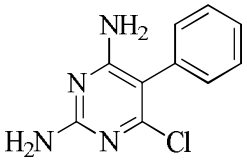
Acetic acid (3.0 mL) and 4-methoxybenzylamine (12.0 g, 87.4 mmol) were added to compound **(83)** (6.00 g, 24.9 mmol) and the mixture was heated at 130°C for 2 h. The cooled residue, in EtOAc, was washed with water and dried. Evaporation and chromatography (EtOAc / petroleum ether 1:9) gave **(84)** (5.8 g, 68%) as a buff oil: ^{1}H NMR ($CDCl_3$) (COSY) δ 3.89 (3 H, s, OMe), 4.48 (2 H, d, *J* = 5.8 Hz, CH_2), 4.80 (1 H, t, *J* = 6 Hz, NH), 4.97 (2 H, br, NH_2), 6.82 (2 H, d, *J* = 8.7 Hz, PMB 3,5-H_2), 7.12 (2 H, d, *J* = 8.7 Hz, PMB 2,6-H_2), 7.25 (2 H, m, Ph 2,6-H_2), 7.36 (1 H, tt, *J* = 7.3, 1.6 Hz, Ph 4-H), 7.43 (2 H, t, *J* = 7.0 Hz, Ph 3,5-H_2); ^{13}C NMR ($(CD_3)_2SO$) (HSQC, HMBC) δ 41.70 (CH_2), 55.19 (Me), 104.30 (pyrimidine 5-C), 112.10 (Ph 1-C), 113.81 (PMB 3,5-C_2), 128.75 (PMB 2,6-C_2), 127.06 (Ph 4-C), 127.78 (Ph 3,5-C_2), 130.92 (Ph 2,6-C_2), 131.60 (PMB 1-C), 134 (Ph 1-C), 150.20 (pyrimidine 6-C), 158.31 (PMB 4-C), 160.36 (pyrimidine 4-C), 161.19 (pyrimidine 2-C), 1 ; MS *m/z* 363.0989 $[M + Na]^+$ [$C_{18}H_{17}{}^{35}ClN_4NaO$ requires 363.0989].

4-Benzylamino-6-chloro-5-phenylpyrimidine-2-amine (86)



Acetic acid (3.0 mL) and benzylamine (12.0 g, 112 mmol) were added to **(83)** (6.00 g, 24.9 mmol) and the mixture was heated at 130°C for 2 h. The cooled residue, in EtOAc, was washed with water. Drying and evaporation gave crude **(86)** (7.5 g, 62%) as a white solid: NMR ($CDCl_3$) δ 4.47 (2 H, d, *J* = 5.7 Hz, CH_2), 4.90 (1 H, br, NH), 5.70 (2 H, br, NH_2), 7.2-7.5 (10 H, m, 2 × Ph-H_5).

6-Chloro-5-phenylpyrimidine-2,4-diamine (87)

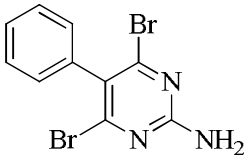


Compound **(84)** (100m g, 0.29 mmol) was stirred with DDQ (130 g, 0.58 mmol) in dichloromethane (0.9 mL) and methanol (0.1 mL) for 4 days, washing out with diethylether and evaporation gave **(87)** (0.03 g, 50%) as white solid, mp >230°C.

^{1}H NMR $(CD_3)_2SO$ δ 6.90 (2 H, br, NH_2), 7.22 (2 H, br, NH_2), 7.32 (2 H, d, *J* = 8.6 Ph 2,6-H_2), 7.55 (3 H, m, Ph 3,4,5-H_3).

MS *m/z*: 243.0390 $[M + Na]^+$ [$C_{10}H_9{}^{35}ClN_4Na$ requires 243.0413].

4,6-Dibromo-5-phenylpyrimidin-2-amine (89)

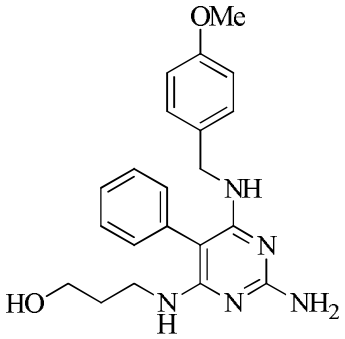


Compound **(81)** (0.50 g, 2.46 mmol) and phosphorus tribromide (1.33 g, 4.91 mmol) were stirred together under reflux for 3 h. Ice was added to the solution and the mixture was stirred for 2 h, then the mixture was neutralised with aq. ammonia (4.0 mL, 35%).

The precipitate was filtered off and dried to give **(89)** (0.72 g, 86 %) as a buff solid: mp >230°C.

^{1}H NMR (COSY) ($CDCl_3$) δ 6.72 (2 H, br, NH_2), 7.07 (1 H, t, *J* = 7.4 Hz, Ph 4-H), 7.24 (2 H, t, *J* = 7.8 Hz, Ph 3,5-H_2), 7.53 (2 H, d, *J* = 7.8 Hz, Ph 2,6-H_2), 10.43 (2 H, br, NH_2); ^{13}C NMR (HMBC, HSQC) δ 123.00 (pyrimidine 5-C), 123.62 (1 C, Ph 4-C), 126.04 (Ph 3,5-C_2), 130.04 (Ph 2,6-C_2), 151.83 (pyrimidine 4,6-C_2), 159.0 (pyrimidine 2-C). MS *m/z* 327.9092 $[M+H]^+$ [$C_{10}H_7N_3{}^{79}Br_2$ requires 327.9084].

6-(3-Hydroxypropylamino)-4-(4-methoxybenzylamino)-5-phenylpyrimidin-2-amine (90).



Method 1

Compound **(84)** (0.10 g, 0.29 mmol) was stirred with 3-aminopropanol (0.11 g, 1.5 mmol) and K_2CO_3 (80 mg, 0.57 mmol) in dry DMF (1.0 mL) at 130°C for 3 d. Evaporation and (EtOAc / MeOH 9:1) gave **(90)** (0.025 g, 21%) as a pale yellow gum.

Method 2

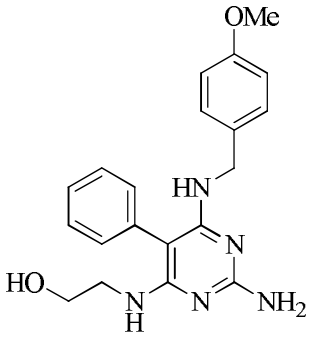
Compound **(84)** (0.07 g, 0.20 mmol) was stirred with 3-aminopropanol (0.04 g, 0.61 mmol) and K_2CO_3 (0.05 g, 0.41 mmol) at 150°C for 3 d. Chromatography (EtOAc / MeOH 9:1) gave **(90)** (0.025 g, 32%) as a pale yellow gum.

^{1}H NMR ($(CD_3)_2SO$) δ 1.34 (2 H, m, propyl 2-H_2), 3.39 (4 H, m, propyl 1,3-H_2), 3.75 (3 H, s, OMe), 4.4 (2 H, d, *J* = 5.7 Hz NHC*H*$_2$Ar), 5.37 (1 H, t, *J* = 6.5 Hz, OH), 5.72 (1

H, t, *J* = 7.0 Hz, NH), 6.86 (2 H, d, *J* = 8.0 Hz, PMB 3,5-H_2), 6.88 (2 H, d, *J* = 8.76 Hz, PMB 2,6-H_2), 7.19 (2 H, t, *J* = 8.9 Hz, Ph 2,6-H_2), 7.26 (1 H, m, Ph 4-H), 7.44-7.53 (2 H, m, Ph 3,5-H_2).

MS *m/z* 380.2119 $[M + H]^+$ [$C_{21}H_{26}N_5O_2$ requires 380.2087], 402.1913 $[M + Na]^+$ [$C_{21}H_{25}N_5NaO_2$ requires 402.1913].

4-(2-Hydroxyethylamino)-6-(4-methoxybenzylamino)-5-phenylpyrimidin-2-amine (91).



Method 1

Compound **(84)** (100 mg, 0.29 mmol) was stirred with K_2CO_3 (500 mg, 3.6 mmol) and 2-aminoethanol (170 mg, 2.8 mmol) in dry DMF (1.0 mL) at 130°C for 3 d under N_2. Evaporation and chromatography (EtOAc / MeOH 9:1) gave **(91)** (30 mg, 30%) as a pale buff gum.

Method 2

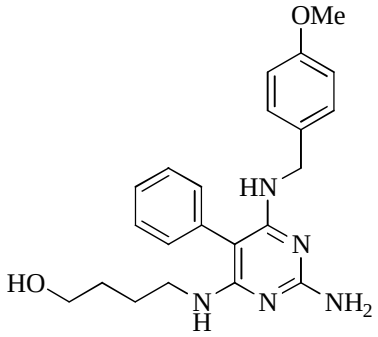
Compound **(84)** (70 mg, 2.05 mmol) was heated with K_2CO_3 (700 mg, 6.15 mmol) and 2-aminoethanol (370 mg, 6.15 mmol) at 150°C for 3 d under N_2. Evaporation and chromatography (EtOAc / MeOH 9:1) gave **(91)** (30 mg, 40%) as a buff gum:

^{1}H NMR (CD_3OD) (COSY) δ 3.60 (2 H, t, *J* = 5.8 Hz, CH_2CH_2ND), 3.87 (2 H, t, *J* = 6.0 Hz, CH_2OD), 3.80 (3 H, *s*, Me), 4.82 (2 H, d, *J* = 6.2 Hz, $NHCH_2Ar$), 6.90 (2 H, d, *J* = 8.7 Hz, PMB 3,5-H_2), 7.20 (2 H, d, *J* = 8.8 Hz, PMB 2,6-H_2), 7.33 (2 H, d, *J* = 6.9 Hz, Ph 2,6-H_2), 7.41 (1 H, t, *J* = 7.4 Hz, Ph 4-H), 7.50 (2 H, t, *J* = 7.3 Hz, Ph 3,5-H_2); ^{13}C NMR (HSQC, HMBC) δ 43.62 (CH_2NH), 43.77 (NCH_2Ar), 55.84 (Me), 60.47 (CH_2OH), 114.32 (PMB 3,5-C_2), 128.02 (pyrimidine 4-C), 129.41 (PMB 2,6-C_2),

129.78 (Ph 2,6-C_2), 130.54 (Ph 3,5-C_2), 132.39 (Ph 4-C), 134.61 (PMB 1-C), 160.49 (PMB 4-C), 160.81 (pyrimidine 5-C), 162.33 (pyrimidine 2-C), 165.80 (pyrimidine 6-C).

MS *m/z* 388.1755 $[M + Na]^+$ [$C_{20}H_{23}N_5O_2$ requires 388.1749].

4-(4-Hydroxybutylamino)-6-(4-methoxybenzylamino)-5-phenylpyrimidin-2-amine (92).



Method 1

Compound **(84**) (100 mg, 0.29 mmol) was stirred with K_2CO_3 (0.08 g, 0.57 mmol) and 4-aminobutanol (0.13 g, 1.46 mmol) in dry DMF (1.0 mL) at 130°C for 3 d. Evaporation and chromatography (EtOAc / MeOH 9:1) gave **(92)** (0.030 g, 26%) as colourless oil.

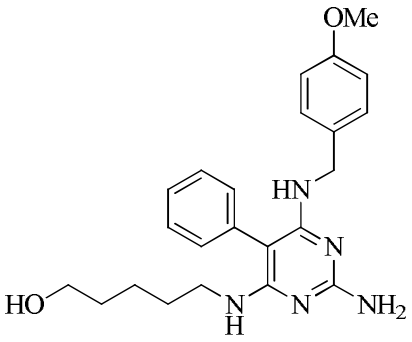
Method 2

Compound **(84)** (100 mg, 0.29 mmol) was heated with K_2CO_3 (0.08 g, 0.57 mmol) and 4-aminobutanol (0.13 g, 1.46 mmol) at 150°C for 3 d. Evaporation and chromatography (EtOAc / MeOH 9:1) gave **(92)** (20 mg, 15%) as a colourless gum.

^{1}H NMR ($(CD_3)_2SO$) δ 1.45 (4 H, m, butyl 2,3-H_4), 3.11 (2 H, q, *J* = .0 Hz, butyl 1-H_4), 3.45 (2 H, t, *J* = 5.8 Hz, butyl 4-H_2), 3.75 (3 H, s, Me), 4.39 (2 H, d, *J* = 5.6 Hz, $ArCH_2NH$), 4.61 (1 H, t, *J* = 6.0 Hz, OH), 5.09 (1 H, t, *J* = 5.5 Hz, NH), 5.60 (2 H, br, NH_2), 6.87 (2 H, d, *J* =8.3 Hz, PMB 3,5-H_2), 7.20 (2 H, d, *J* = 8.6 Hz, PMB 2,6-H_2), 7.24 (2 H, d, *J* = 6.8 Hz, Ph 2,6-H_2), 7.39 (1 H, t, *J* = 7.5 Hz, Ph 4-H), 7.50 (2 H, t, *J* = 7.4 Hz, Ph 3,5-H_2).

MS *m/z* 416.2091 [M + Na]$^+$, [$C_{22}H_{27}N_5NaO_2$ requires 416.2062].

4-(5-Hydroxypentylamino)-6-(4-methoxybenzylamino)-5-phenylpyrimidin-2-amine (93).



Method 1

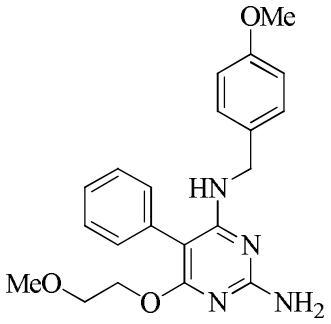
Compound **(84)** (100 mg, 0.29 mmol) was heated with K_2CO_3 (50 mg, 0.41 mmol) and 5-aminopentanol (150 mg, 1.45 mmol) dry DMF (1.0 mL) at 130°C for 3 d. Evaporation and chromatography (EtOAc / MeOH 9:1) gave **(93)** (20 mg, 19%) as a colourless gum.

Method 2

Compound **(84)** (70 mg, 0.20 mmol) was heated with K_2CO_3 (50 mg, 0.36 mmol) and 5-aminopentanol (60 mg, 0.58 mmol) at 150°C for 3 d under N_2. Evaporation and chromatography (EtOAc / MeOH 9:1) gave **(93)** (23 mg, 28%) as a colourless gum: ^{1}H NMR ($(CD_3)_2SO$) (COSY) δ 1.19 (3 H, q, *J* = 6.5 Hz, pentyl 3-H_2), 1.36 (3 H, q, *J* = 6.0 Hz, pentyl 4-H_4), 3.17 (2 H, qr, *J* = 7.0 Hz, pentyl 1-H_2), 3.34 (2 H, t, J = 7.0 Hz, pentyl 5-H_2), 3.69 (3 H, s, Me), 4.35 (2 H, d, *J* = 6.5 Hz, ArC*H*$_2$NH), 4.54 (1 H, t, *J* = 6.0 Hz, OH), 5.04 (1 H, t, *J* = 6.0 Hz, NH), 5.60 (2 H, br, NH_2), 6.81 (2 H, d, *J* = 6.5 Hz, PMB 3,5-H_2), 7.14 (2 H, d, *J* = 9.0 Hz, PMB 2,6-H_2), 7.19 (2 H, d, *J* = 8.0 Hz, Ph 2,6-H_2), 7.33 (1 H, t, *J* = 7.4 Hz, Ph 4-H), 7.46 (2 H, t, *J* = 7.5 Hz, Ph 3,5-H_2); ^{13}C NMR (HSQC, HMBC) δ 11.87 (pentyl 3-C), 29.49 (pentyl 2-C), 32.26 (pentyl 4-C), 41.0 (pentyl 1-C), 60.64 (pentyl 5-C), 54.95 (Me), 42.78 (CH_2PMB), 113.44 (PMB 3,5-C_2), 127.06 (pyrimidine 4-C), 128.26 (PMB 2,6-C_2), 129.61 (Ph 2,6-C_2), 131.48 (Ph3,5-C_2), 133.26 (Ph 4-C), 134.00 (PMB 1-C), 157.83 (PMB 4-C), 159.49 (pyrimidine 5-C), 159.93 (pyrimidine 2-C), 161.53 (pyrimidine 6-C);

MS *m/z* 408.2496 $[M + H]^+$ [$C_{23}H_{30}N_5O_2$ requires 408.2400].

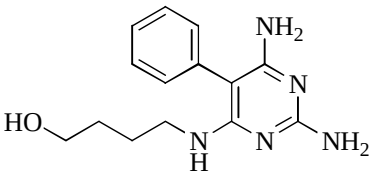
Formation of 4-(2-methoxyethoxy)-6-(4-methoxybenzylamino)-5-phenylpyrimidin-2-amine (94).



Compound **(84)** (0.10 g, 0.29 mmol) was heated with K_2CO_3 (80 mg, 0.57 mmol) and 4-aminobutanol (130 mg, 1.5 mmol) in 2-methoxyethanol (1.0 mL) at 130°C for 3 d. Evaporation and chromatography (EtOAc / MeOH 9:1) gave **(94)** (0.05 mg, 45 %) as a white solid: mp 85-87°C; ^{1}H NMR ($(CD_3)_2SO$) δ 3.22 (3 H, s, CH_2OMe), 3.52 (2 H, t, *J* = 5.1 Hz, CH_2OMe), 3.75 (3 H, *s*, ArOMe), 4.30 (2 H, t, *J* = 4.9 Hz, CH_2O-pyrimidine), 4.45 (2 H, d, *J* = 6.2 Hz, $NHCH_2Ar$), 5.83 (1 H, t, *J* = 5.9 Hz, NH), 6.1 (2 H, br, NH_2), 6.88 (2 H, d, *J* = 8.5 Hz, PMB 3,5-H_2), 7.20-7.30 (5 H, m, PMB 2,6-H_2, Ph 2,4,6-H_3), 7.42 (2 H, t, *J* = 7.5 Hz, Ph 3,5-H_2).

MS *m/z* 403.1765 $[M + Na]^+$ [$C_{21}H_{24}N_4NaO_3$ requires 403.1746].

4-((2,6-Diamino-5-phenylpyrimidin-4-yl)amino)butan-1-ol (95)



Method 1

Compound **(87)** (0.34 g, 0.67 mmol) was stirred with K_2CO_3 (60 mg, 0.45 mmol) and 4-aminobutanol (5 mg, 0.22 mmol) in dry DMF (1.0 mL) at 130°C for 3 d. The mass spectrum showed the compound **(95)** but it could not be isolated from the mixture.

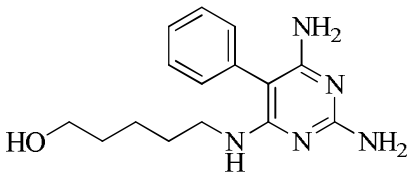
Method 2

DDQ (20 mg, 0.1 mmol) was stirred with **(92)** (20 mg, 0.05 mmol) in dichloromethane (0.9 mL) and methanol (0.1 mL) for 4 d. The compound **(95)** could not be isolated from the mixture but it was identifiable by NMR and mass spectroscopy.

^{1}H NMR ($(CD_3)_2SO$) δ 1.45 (4 H, m, butyl 2,3-H_4), 3.0-3.9 (4 H, m, butyl 1,4-H_4), 5.8 (2 H, br, NH_2), 7.20 (2 H, d, *J* = 7.28 Hz, Ph 2,6-H_2), 7.60 (3 H, m, Ph 3,4,5-H_3).

MS *m/z* 247.1659 $[M + H]^+$ [$C_{14}H_{20}N_5O$ requires 274.1668].

5-((2-Amino-6-5-phenylpyrimidin-4-yl)amino)pentan-1-ol (96)

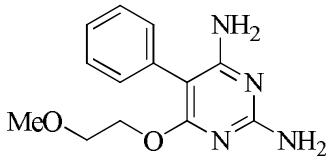


DDQ (20 mg, 80 μmol) was stirred with **(93)** (20 mg, 40 μmol) in CH_2Cl_2 (0.9 mL) and MeOH (0.1 mL) for 4 d. Compound **(96)** could not be isolated from the mixture but it was identified by NMR and mass spectroscopy.

^{1}H NMR ($(CD_3)_2SO$) δ 1.28 -1.45 (6 H, m, pentyl 2,3,4-H_6), 3.10-3.9 (4 H, *m*, pentyl 1,5-H_4), 7.26 (2 H, d, *J* = 8.6 Hz, Ph 2,6-H_2), 7.50 (1 H, t, *J* = 7.3 Hz, Ph 4-H), 7.60 (2 H, t, *J* = 7.8 Hz, Ph 3,5-H_2).

MS *m/z* 288.1802 $[M + Na]^+$ [$C_{15}H_{22}N_5O$ requires 288.1824].

6-(2-Methoxyethoxy)-5-phenylpyrimidine-2,4-diamine (97)

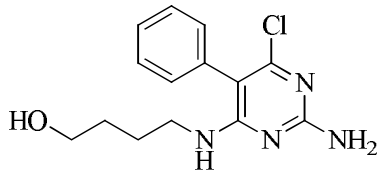


Compound **(94)** (0.100 g, 0.26 mmol) was stirred with DDQ (0.11 g, 0.52 mmol) in dichloromethane (0.9 mL) and methanol (0.1 mL) for 4 d. Evaporation of the solvent and washing the residue with diethyl ether gave **(97)** (0.045 g, 70%) as a pale yellow gum.

^{1}H NMR ($(CD_3)_2SO$) δ 3.22 (3 H, s, Me), 3.55 (2 H, t, *J* = 4.6 Hz, C*H*$_2$OMe), 4.40 (2 H, t, *J* = 4.9 Hz, CH_2O-pyrimidine), 5.80 (2 H, br, NH_2), 7.20 (2 H, d, *J* = 7.0 Ph 2,6-H_2), 7.38(1 H, t, *J* = 7.2 Hz, Ph 4-H), 7.50 (2 H, t, *J* = 7.2 Hz, Ph 2,6-H_2).

MS *m/z* 283.1171 $[M + Na]^+$ [$C_{13}H_{16}N_4NaO_2$ requires 283.1170].

4-((2-Amino-6-chloro-5-phenylpyrimidin-4-yl)amino)butan-1-ol (98)



K_2CO_3 (0.56 g, 4.1 mmol) was stirred with (**83)** (1.0 g, 4.2 mmol) and 4-aminobutanol (0.37 g, 4.2 mmol) in EtOH (5.0 mL) at 60°C for 16 h. Evaporation and chromatography (EtOAc/ MeOH 9:1) gave **(98)** (0.67 g, 55%) as a white solid.

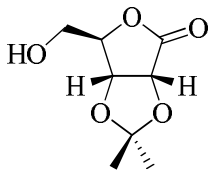
^{1}H NMR ($(CD_3)_2SO$) (COSY) δ 1.39 (2 H, qn ,*J* = 7.4 Hz, butyl 3-H_2), 1.49 (2 H, qn, *J* = 7.0 Hz, butyl 2-H_2), 3.28 (2 H, q, *J* = 6.3 Hz, butyl 1-H_2), 3.40 (2 H, q, *J* = 5.3 Hz, butyl 4-H_2), 4.36 (1 H, t, *J* =5.1 Hz, OH), 5.60 (1 H, br, NH), 6.45 (2 H, s, NH_2), 7.25

(2 H, d, J = 6.8 Hz, Ph 2,6-H_2), 7.44 (1 H, t, J = 7.3 Hz , Ph 4-H), 7.50 (2 H, t, J = 7.0 Hz, Ph 3,5-H_2).

^{13}C NMR (HMBC) (HSQC) δ 25.61 (1 C, *C*H_2CH$_2$NH), 29.82 (1 C, *C*H_2CH$_2$OH), 41.0 (1 C, *C*H_2OH), 60.0 (1 C, *C*H_2NH), 105 (1 C, *C*NH_2), 127.71 (1 C, Ph 4-C), 128.90 (2 C, Ph 3.5-C_2), 130.82 (2 C, Ph 2 ,6-C_2), 133,0 (1 C, Ph 1-C), 151 (1 C, C-Cl). M.p 145-147°C.

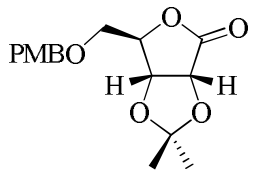
MS *m/z*: 293.1178 $[M + H]^+$ [$C_{14}H_{18}{}^{35}ClN_4O$ requires 293.1169].

3a*R*,6*R*,6a*R*-2,2-Dimethyl-6-hydroxymethyldihydrofuro[3,4-*d*][1,3]dioxol-4(3*H*)-one[76] (112)



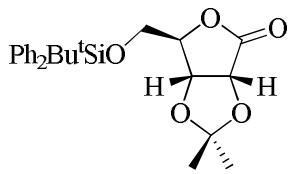
A mixture of D-ribonolactone (4.35 g, 29.3 mmol), 2,2-dimethoxypropane (15.2 g, 146 mmol) and pyridinium 4-methylbenzenesulfonate (195 mg, 0.79 mmol) was heated at 60°C for 4 h; excess reagent was then evaporated. The residue, in EtOAc, was washed with sat. aq. $NaHCO_3$ and dried. The evaporation residue was dissolved in THF (15.0 mL) and aq. HCl (0.1 M, 10.0 mL) and the mixture was stirred for 10 min at room temperature. The solvent was evaporated and the residue was dissolved in EtOAc. This solution was washed with sat. aq. $NaHCO_3$, then dried. Evaporation gave **(111)** (3.00 g, 62%) as a white solid: mp 135-137°C (lit.[76] mp 135-137°C); ^{1}H NMR ($CDCl_3$) (COSY , NOESY) δ 1.42 (3 H, s, Me_{endo}), 1.52 (3 H, s, Me_{exo}), 3.84 (1 H, dd, J = 12.1, 1.8 Hz, HOC*H*), 4.02 (1 H, dd, J = 12.1, 2.1 Hz, HOC*H*), 4.65 (1 H, t, J = 2.1 Hz, 6-H), 4.81 (1 H, d, J = 5.6 Hz, 6a-H), 4.86 (1 H, d, J = 5.6 Hz, 3a-H); ^{13}C NMR ($CDCl_3$) (HSQC , HMBC) δ 25.48 (Me_{endo}), 26.71 (Me_{exo}), 62.11 (CH_2OH), 75.59 (3a-C), 78.20 (6a-C), 82.43 (6-C), 113.18 (2-C), 174.59 (4-C).

3a*R*,6*R*,6a*R*)-6-((4-methoxybenzyloxy)methyl)-2,2-dimethyldihydrofuro[3,4-*d*][1,3]dioxol-4(3a*H*)-one[78] (113)



Compound **(112)** (5.00 g, 21.1 mmol) and NaH (60% in oil, 1.27 g, 32 mmol) were mixed together in dry DMF (18.3 mL), then 4-methoxybenzyl chloride (5.00 g, 32 mmol) was added and the mixture was stirred overnight. The evaporation residue, in diethyl ether, was washed with water. Drying, evaporation and chromatography (petroleum ether/ EtOAc) (3:1) gave **(113)** (1.63 g, 24%) as pale yellow oil (lit.[78] oil): ^{1}H NMR ($CDCl_3$) δ 1.28 (3 H, s, Me), 1.38 (3 H, s, Me), 3.55 (1 H, dd, *J* = 10.4, 2.4 Hz, PMBOC*H*), 3.59 (1 H, dd, *J* = 10.4, 2.4 Hz, PMBOC*H*), 3.72 (3 H, s, Me), 4.32 (1 H, d, *J* = 12.4 Hz, ArCH), 4.40 (1 H, d, *J* = 12.4 Hz, ArCH), 4.53 (1 H, t, *J* = 2.3 Hz, 6-H), 4.60 (1 H, d, *J* = 6.4 Hz, 6a-H), 4.66 (1 H, d, *J* = 6.4 Hz, 3a-H), 6.80 (2 H, d, *J* = 9.2 Hz, Ar 3,5-H_2), 7.10 (2 H, d, *J* = 8.8 Hz, Ar 2,6-H_2); ^{13}C NMR ($CDCl_3$) δ 25.55 (Me), 26.72 (Me), 55.22 (OMe), 68.65, 73.50, 75.65, 78.34, 81.07, 113.05 (Ar 1-C), 113.93 (Ar 3,5-C_2), 129.27 (Ar 2,6-C_2), 159.51 (Ar 4-C), 174.24 (C=O).

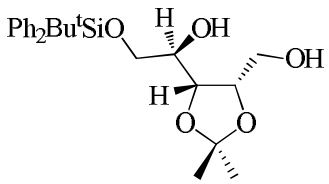
(3a*R*,6*R*,6a*R*)-6-((*tert*-Butyldiphenylsilyloxy)methyl)-2,dimethyldihydrofuro[3,4-*d*][1,3]dioxol-3(3a*H*)-one[79] (114)



Compound **(112)** (3.00 g, 15.9 mmol), imidazole (2.00 g, 29.3 mmol) and anhydrous DMF (20 mL) were mixed; *tert*-butyldiphenylsilyl chloride (4.20 g, 14.5 mmol) was added and the mixture was stirred at room temperature overnight. The reaction mixture was poured into water and extracted with diethyl ether. Drying and evaporation gave

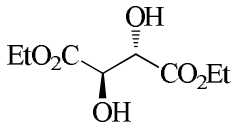
(114) (4.20 g, 62%) as a white solid: mp 77-80°C (lit.[85] mp 92-94°C); ^{1}H NMR ($CDCl_3$) (COSY , NOESY) δ 1.04 (9 H, s, Bu^t), 1.40 (3 H, s, Me_{endo}), 1.49 (3 H, s, Me_{exo}), 3.75 (1 H, dd, *J* = 11.5, 1.5 Hz, SiOCH), 3.91 (1 H, dd, *J* = 11.5, 2.5 Hz, SiOCH), 4.58 (1 H, t, *J* = 1.8 Hz, 6-H), 4.73 (1 H, d, *J* = 5.6 Hz, 6a-H), 4.90 (1 H, d, *J* = 5.6 Hz, 3a-H), 7.35-7.49 (6 H, m, Ph), 7.60-7.64 (3 H, Ph), 7.72 (1 H, dd, *J* = 8.0, 2.0 Hz, Ph); ^{13}C NMR ($CDCl_3$) (HSQC, HMBC) δ 25.5 (Me_{endo}), 26.50 (Me_{exo}), 26.69 (Me_3), 29.69 (C-Si), 63.54 (CH_2O), 75.8 (3a-C), 78.4 (6a-H), 82.32 (6-C), 127.70 (2-C), 128.00 (2C-Ar), 129.64 (C-Ar), 130.17 (C-Ar), 131.52 (C-Ar), 132.35 (C-Ar), 134.78 (C-Ar), 135.43 (2C-Ar), 135.60 (2C-Ar), 174.09 (4-C).

(*R*)-2-(*tert*-butyldiphenylsilyloxy)-1-((4*R*,5*S*)-5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)ethanol (115)



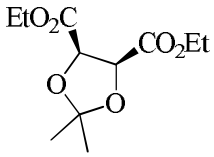
Compound **(114)** (3.00 g, 7.03 mmol) in dry EtOH (25 mL) at 0°C was stirred and $NaBH_4$ (400 mg, 10.6 mmol) was added. The mixture was stirred for 2 h. NH_4Cl (500 mg, 9.3 mmol) was added and the mixture was stirred for 5 min, then filtered and evaporated to give an oil, which was purified by chromatography (EtOAc / petroleum ether 1:9) to give **(115)** (2.7 g, 89%) as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 1.18 (9 H, s, Bu^t), 1.28 (3 H, s, Me), 1.30 (3 H, s, Me), 3.06 (1 H, br, 2 × OH), 3.75 (1 H, dd, *J* = 10.5, 6.5, SiOCH), 3.75-3.90 (3 H, m, C*H*$_2$OH + C*H*OH), 3.90 (1 H, dd, *J* = 10.5, 3.0 Hz, SiOCH), 4.11 (1 H, dd, *J* = 9.5, 6.0 Hz, 4-H), 4.35 (1 H, dt, *J* = 7.5, 5.5 Hz, 5-H), 7.39-7.67 (10 H, 2 × Ph); ^{13}C NMR ($CDCl_3$) (HSQC , HMBC) δ 19.29 (C-Si), 25.22 (Me), 26.87 (Me_3), 27.74 (Me), 60.86 (CH_2OH), 65.34 (CH_2OSi), 69.68 (CHOH), 76.53 (5-C), 77.56 (4-C), 108.53 (2-C), 127.82 (Ph 3,5-C_2), 127.86 (Ph' 3,5-C_2), 129.29 (Ph 4-C), 129.97 (Ph' 4-C), 132.78 (Ph 2,6-C_2), 132.90 (Ph' 2,6-C_2).

Diethyl -2,3-dihydroxybutanedioate[45] (116)



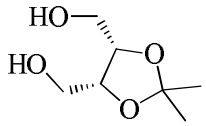
meso-Tartaric acid (1.00 g, 6.7 mmol) was boiled under reflux with 4-methyl-benzenesulfonic acid (2.30 g, 13.3 mmol) in EtOH (8.0 mL) for 16 h. The solvent was evaporated. The residue, in ethyl acetate, was washed with water and dried. The solvent was evaporated to give **(116)** (0.80 g, 58%) as white crystals: ^{1}H NMR ($CDCl_3$) (COSY) δ 1.30 (6 H, m, 2 Me), 3.82 (2 H, s, 2 × OH), 4.15-4.30 (4 H, m, 2 × CH_2), 4.80 (2 H, s, 2 × C*H*OH); ^{13}C NMR (HSQC, HMBC) δ 14.03 (2 × Me), 62.29 (2 × CH_2), 72.81 (2 × CHOH), 171.01 (2 × C=O).

Diethyl *R,R*-2,2-dimethyl-1,3-dioxolane-4,5-dicarboxylate[45](117)



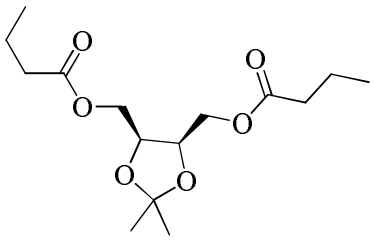
Compound **(116)** (1.00 g, 4.66 mmol) was stirred with 2,2-dimethoxypropane (0.53 g, 5.3 mmol) and 4-methylbenzenesulfonic acid (4.4 mg, 0.02 mmol) in toluene (50 mL) at 80°C with 4 Á molecular sieves (0.5 g) for 16 h. Na_2CO_3 (5.0 mg, 0.06 mmol) was added. The mixture was filtered and dried and the solvent was evaporated to give **(117)** (0.98 g, 86%) as colourless oil (lit.[86] oil): ^{1}H NMR ($CDCl_3$) δ 1.25 (6 H, t, *J* = 7.2 Hz, 2 × CH_2CH_3), 1.38 (3 H, s, C-Me), 1.62 (3 H, s, C-Me), 4.17 (4 H, m, 2 × C*H*$_2$CH$_3$), 4.78 (2 H, s, 4,5-H_2).

_S,S_-4,5-Di(hydroxymethyl)-2,2,dimethyl-1,3-dioxolane[45] (118)



$LiAlH_4$ (0.33 g, 8.25 mmol) in dry THF (3.3 mL) was heated for 30 min; **(117) (**1.0 g, 8.3 mmol) was added and the mixture was heated under reflux for 5 h. After cooling, water (3.0 mL), aq. NaOH (4 M, 3.0 mL) and water (9 mL) were added. Filtration and evaporation gave **(118)** (0.74 g, 55%) as a pale yellow oil (lit.[87] oil): ^{1}H NMR ($CDCl_3$) δ 1.38 (3 H, s, Me), 1.62 (3 H, s, Me), 3.70 (2 H, dd, *J* = 11.5, 6.1 Hz, 2 × C*H*HOH), 3.80 (2 H, dd, *J* = 11.5, 5.2 Hz, 2 × CH*H*OH), 3.90 (2 H, br, 2 × OH), 4.30 (2 H, t, *J* = 4 Hz, 4,5-H_2); ^{13}C NMR (HSQC, HMBC) δ 25.60 (C-Me_{endo}), 27.49 (C-Me_{exo}), 61.59 (4,5-C_2), 78.90 (2 × CH_2OH), 109.55 (2-C).

_S,S_-4,5-Di(butanoyloxymethyl)-2,2,dimethyl-1,3-dioxolane (119)



To **(118)** (1.0 g, 5.0 mmol) in dichloromethane (5.0 mL), butanoyl chloride (1.17 g, 11 mmol) in dichloromethane (2.0 ml) was added, followed by triethylamine (2.50 g, 24 mmol). The mixture was stirred for 16 h. The solvent was evaporated. The residue, in dichloromethane, was washed with water and dried. Evaporation and chromatography (EtOAc / petroleum ether) (1:3) gave **(119)** (1.4 g, 65%) as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 0.96 (6 H, t, *J* = 7.4 Hz, 2 × butanoyl 4-H_3), 1.36 (3 H, s, C-Me), 1.47 (3 H, s, C-Me), 1.65 (4 H, sextet, *J* = 7.4 Hz, 2 × butanoyl 3-H_2), 2.43 (4 H, t, *J* = 7.5 Hz, 2 × butanoyl 2-H_2), 4.1 (2 H, dd, *J* = 11.6, 6.4 Hz, 2 × C*H*HO_2CPr), 4.28 (1 H, dd, *J* = 11.0, 6.0, Hz, 2 × CH*H*O_2CPr), 4.36 (2 H, t, *J* = 7.0 Hz, 4,5-H_2).

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